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A CLINICAL, SEROLOGICAL AND PATHOLOGICAL STUDY OF CONCURRENT ANAPLASMOSIS AND BABESIOSIS IN EXPERIMENTALLY INFECTED CALVES

A Thesis

by

DONALD EARL CORRIER

Submitted to the Graduate College of
Texas A&M University in
partial fulfillment of the requirement for the degree of

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A CLINICAL, SEROLOGICAL AND PATHOLOGICAL STUDY OF CONCURRENT ANAPLASMOSIS AND BABESIOSIS IN EXPERIMENTALLY INFECTED CALVES

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ABSTRACT

A Clinical, Serological and Pathological Study of Concurrent Anaplasmosis and Babesiosis in Experimentally Infected Calves

(December 1972)

Donald Earl Corrier, B.A., Eastern Washington College;

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Directed by: Dr. Charles H. Bridges

Isolates of Anaplasma marginale and of Babesia bigemina were obtained from naturally infected calves with mixed infections of A. marginale, B. bigemina and B. argentina. Babesia bigemina was separated from B. argentina and A. marginale by rapid serial passage through 5 splenectomized calves. Anaplasma marginale was separated from B. bigemina and B. argentina by administering a sterilizing dose of a babesiacidal drug. Following the separation procedures, the isolates of A. marginale and B. bigemina were frozen and stored at -79 C.

Twenty-two, 7 month old, male, nonsplenectomized Holstein-Friesian calves were divided into 4 experimental groups. Group I and II consisted of 3 calves each and served as control groups to study anaplasmosis and babesiosis respectively. Groups III and IV consisted of 8 calves each and served to study a concurrent infection with B. bigemina in calves recovering from anaplasmosis and a concurrent infection with A. marginale in calves recovering from babesiosis respectively. The calves were infected by subcutaneous

inoculation. Calves infected with A. marginale received approximately $9.5x10^8$ organisms. Calves infected with B. bigemina received approximately $10.5x10^8$ organisms.

Clinical manifestations of disease were mild and consisted of slight fever, poor body condition, decreased weight gain and inactivity. Fever was routinely observed only in the calves infected with A. marginale. The clinical signs of disease observed in the concurrently infected calves were attributed predominately to infection with A. marginale.

Associated with the appearance of parasitized erythrocytes were decreases in packed cell volume, hemoglobin, albumin:globulin ratio, and serum albumin, and slight increases in the levels of serum bilirubin, serum glutamic oxalacetic transaminase, and alpha and beta serum globulins. Decreases in PCV and hemoglobin concentration were more prolonged and severe in the concurrently infected calves and were considered to have resulted predominately from infection with A. marginale. Changes observed later in the experiment included the appearance of reticulocytes in the blood, a decrease in myeloid:erythroid ratio in the bone marrow, and an increase in the levels of total serum proteins, serum gamma globulins and serum sorbital dehydrogenase. A prolonged monocytosis occurred in each of the 4 groups of calves.

Gross lesions observed in the concurrently infected calves included a moderately excessive quantity of yellow fluid in the

peritoneal and pleural cavities, moderate lymph node enlargement, splenomegaly and hepatomegaly, moderate renal congestion, and occasional serous atrophy of depot fat.

Hepatocellular degeneration and necrosis were observed in the centrolobular areas of the liver. Lymphoid hyperplasia was observed in the malpighian corpuscles of the spleen and in the lymphoid follicles of the lymph nodes. Hemosiderosis of the spleen, liver, kidney and lymph nodes was attributed to the increased removal of damaged erythrocytes from the circulation with the subsequent release of breakdown products of hemoglobin.

Complement fixing antibodies for Anaplasma were first observed on days 17 to 26 postinoculation and corresponded to the increase in the alpha and beta globulins. Increases in complement fixing antibody titer corresponded to the increase in gamma globulin and persisted until the concentration of gamma globulin began to recede during the terminal days of the experiment.

Complement fixing antibodies for Babesia were first observed on day 12 postinoculation, which corresponded to the increase in gamma globulin. The increase and decrease in antibody titer for babesiosis corresponded to the increase and decrease in gamma globulin.

The biological relationship of A. marginale and B. bigemina during the concurrent infection appeared to be one of independency. Neither an inhibitory nor a synergistic relationship was apparent

during the investigation. The clinical and pathological manifestations of concurrent infection were more severe than those observed during infection with either of the hemotrophic parasites alone, and were attributed to the concurrent infection being additive in nature.

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CHAPTER I

INTRODUCTION

Anaplasmosis and babesiosis are infectious, tick-borne diseases caused by hemoparasites. Although several domestic and wild animal species are susceptible to the infection caused by Anaplasma and Babesia organisms, economic losses are most severe in cattle.[61,63] The incidence of bovine anaplasmosis and babesiosis is especially high in the tropical and subtropical regions of the world where large enzootic areas exist. Indigenous cattle within the enzootic areas are normally exposed to infection at an early age when their resistance to the disease is reported to be highest.[61,62,63] The indigenous cattle usually display minimal signs of a clinical infection and subsequently develop a chronic carrier state of infection through a process of natural premunition.[61,62,63] However, cattle which are transported from non-enzootic areas into or through enzootic areas for the purpose of shipment to market, utilization of pasture land or as imported breeding stock are highly susceptible to severe clinical infection with high mortality rates often occurring.[61,62,63]

Numerous investigators have commented on the common distribution of anaplasmosis and babesiosis and on the high frequency of concurrent Anaplasma sp. and Babesia sp. infection of cattle.[7,11,18,44,50,52,54,72,81,82,86,87] Several investigators have observed an

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The citations in this thesis follow the style of the American Journal of Veterinary Research.

increased severity in clinical illness in cattle concurrently infected.[18,53,82] Others have reported the results of experiments which indicate that either of the disease agents may initiate in the bovine host a physiological state which is not conducive to the multiplication of the other disease agent.[52,83]

The purpose of this investigation was to study the clinical course, serological response and pathological manifestations of cattle concurrently infected with Anaplasma marginale and Babesia bigemina, and to study the biological behavior of the 2 disease agents during a concurrent infection.

CHAPTER II

REVIEW OF LITERATURE

Anaplasma sp. and *Babesia* sp. are hemotropic parasites which invade the erythrocytes of both domestic and wild animals. *Babesia* organisms were first described in the erythrocytes of cattle in 1888.[37,58,61,75] Shortly thereafter, *Babesia bigemina* was described during the investigations into the nature and cause of Texas Fever in the United States.[75] The authors observed intra-erythrocytic "peripheral coccus-like bodies" which they considered to be a stage in the life cycle of *B. bigemina*. The investigators also reported cases in which cattle with Texas Fever passed through relapses or mild secondary attacks during which the peripheral coccus-like body constantly appeared in the erythrocytes. A series of experiments from 1910 to 1912, demonstrated that the intra-erythrocytic coccoid bodies described by previous investigators were not associated with *Babesia*, but represented a new genus of protozoa which was named *Anaplasma marginale*.[79,80]

Several authors have reviewed and summarized the literature on anaplasmosis[58,62,63] and on babesiosis.[37,58,61] In addition several textbooks have sections devoted to anaplasmosis[6,7,12,30,68] and to babesiosis.[6,7,11,30,68]

The classification of *Anaplasma* and of *Babesia* species has been rather uncertain. *Babesia* were classified in the Phylum Protozoa since their earliest description, however, their subclassification

within this Phylum was difficult due to the uncertain relationship of the Babesia to other protozoa.[51,61] The uncertainty stems from the lack of knowledge of the complete life cycle of the Babesia in the vertebrate and invertebrate hosts.[51] Anaplasma species were reported to be more closely related to the Rickettsiae.[48,62] However, the close resemblance of anaplasmosis to a protozoan disease has often resulted in the Anaplasma being called protozoan-like agents.[32]

Anaplasmosis and babesiosis have been studied extensively as single disease entities of cattle. Numerous investigators have described the clinical signs and gross lesions associated with bovine anaplasmosis.[7,11,28,30,36,45,62,72,73,74] The descriptions included: fever; anorexia; dehydration; emaciation; depression; weakness; constipation; anemia; pale to icteric mucus membranes; leukocytosis; splenomegaly; hepatomegaly with yellow discoloration; general lymph node enlargement; distention of the gall bladder; serous atrophy of renal and cardiac fat; occasional petechial hemorrhages in the pleura, epicardium and pericardium; red bone marrow in the medullary cavity of the long bones; and serous effusions in the body cavities. The clinical course of anaplasmosis was divided into 4 stages:[62] (1) a prepatent stage of 20 to 40 days; (2) a stage of increasing anemia; (3) a stage of maximum anemia; (4) a stage of convalescence.

Histopathological lesions occurring in anaplasmosis have been described by several investigators.[1,28,30,45,64,73,74] The extent and severity of the microscopic lesions varied according to the stage of the infection and included: extramedullary hematopoiesis;

splenomegaly with slight to marked lymphoid hyperplasia of the splenic corpuscles, hemosiderosis, erythrophagocytosis and an increase in reticuloendothelial macrophages in the red pulp of the spleen; hypertrophy of the Kupffer cells of the liver with many of the cells containing phagocytized erythrocytes and hemosiderin; occasional hepatic centrolobular degeneration and bile retention in the biliary canaliculi; lymphoid hyperplasia in the mesenteric, hepatic, mediastinal and bronchial lymph nodes with other lymph nodes also being affected; an increase in reticuloendothelial macrophages in the sinuses of the above mentioned lymph nodes with hemosiderosis and erythrophagocytosis also being present; and an increase in the cellularity of the renal glomeruli with degenerative changes in the epithelial cells of the renal tubules.

The clinical signs and gross lesions associated with bovine babesiosis have been described by numerous authors[7,10,11,30,61,66,72,74,75] and included: fever of 40 to 41 C; anorexia; pale to icteric mucus membranes; anemia; hemoglobinuria; weakness; convulsions and dilirium in occasional cases; weight loss; splenomegaly with the spleen having a soft pulpy consistency; hepatomegaly with yellow to orange discoloration and biliary stasis; excessive granular bile in gall bladder; hydropericardium; petechial to ecchymotic hemorrhages in the epicardium and endocardium; marked icterus of serosal surfaces in acute cases; swollen congested kidneys; catarrhal gastro-enteritis in occasional cases; serous atrophy of depot fat and edema of the connective tissues in prolonged cases.

Histopathological lesions described in bovine babesiosis were reported to include: hepatic parenchymatous degeneration and biliary stasis; pulmonary edema; hemoglobin within the renal tubules with degenerative changes in the tubular epithelium; splenic congestion with lymphoid hypertrophy in the splenic corpuscles; and obstruction of capillaries in various organs due to numerous Babesia parasites.[11,19,30,72] In cattle with central nervous system signs, the histopathological lesions included perivascular, perineural and interstitial edema of the brain associated with congested cerebral capillaries due to obstruction with erythrocytes containing Babesia parasites.[10,19,30,72]

Anemia due to the excessive destruction of the host's erythrocytes is a constant finding in both acute bovine anaplasmosis and babesiosis. The excessive release of intraerythrocytic contents and the hypoxic state which accompanies the anemic condition are responsible for many of the clinical signs and pathological alterations which accompany anaplasmosis and babesiosis. In babesiosis, anemia is the result of the intravascular hemolysis of parasitized erythrocytes.[30,61] In anaplasmosis, the anemia observed is initially associated with the removal of parasitized erythrocytes by the host's reticulo-endothelial system.[1,30,45] However, during anaplasmosis the period of maximum anemia is often observed to occur 1 to 5 days following the height of parasitemia.[28,62] A close correlation between the number of erythrocytes parasitized and the number of erythrocytes removed from the circulation does not always exist. The removal of

non-parasitized erythrocytes by the host's reticuloendothelial system during anaplasmosis has been attributed to an auto-antibody mechanism.[13,25,26,27,28,29,35,43,46,47,62,69,70,71]

The common distribution of anaplasmosis and babesiosis and the high incidence of concurrent infection of cattle with both Anaplasma and Babesia species was reported by numerous investigators as incidental findings[7,11,18,44,50,54,72,81,82,86,87] and as investigations dealing directly with concurrent infections.[52,83] The common occurrence of anaplasmosis and babesiosis has resulted in the practice of premunizing cattle against both of the diseases prior to their movement into enzootic areas.[34,44,50,81,82,86]

The common distribution of anaplasmosis and babesiosis appears to be due to ecological factors which favor the survival and prevalence of tick vectors. Ticks have been shown to be the principal vectors responsible for the transmission of both A. marginale and B. bigemina under natural field conditions.[7,58,61,62] Some 20 species of ticks were reported to be capable of transmitting Anaplasma.[62] At least 5 species of ticks are capable of transmitting Babesia.[61]

Descriptions of the clinical nature of concurrent anaplasmosis and babesiosis have been few and have consisted primarily of reports of observations made during premunition studies. Clinical signs of anaplasmosis were observed to occur 3 to 6 weeks following the clinical phase of babesiosis.[7,11] The 3 to 6 week delay in the appearance of the clinical signs of anaplasmosis was attributed to

the difference in the prepatent periods for the 2 diseases. Anaplasmosis has a prepatent period of 20 to 40 days,[62,63] while the prepatent period for babesiosis, due to B. bigemina, is reported to be from 5 to 10 days.[61]

When premunizing against both anaplasmosis and babesiosis, it was reported to be more practical to premunize against babesiosis first, followed in 3 weeks by premunization against anaplasmosis.[18, 82] The procedure was suggested as a means of reducing the severity of the clinical reaction which was observed to accompany simultaneous premunization with both Anaplasma and Babesia species. The severe clinical response and rather high mortality rate which was observed to accompany simultaneous premunization was attributed to an intense anemia.[18,82] The authors attributed the severe anemia to the additive effect of the individual anemias caused by each of the 2 disease agents.

The biological behavior of the Anaplasma and Babesia organisms during concurrent and intercurrent infections has not been clearly explained. Acute clinical exacerbations of anaplasmosis were reported to be especially common following secondary infection with other protozoan organisms which cause anemia and place additional stress on the reticuloendothelial system of the host.[30,56] Following infection with rinderpest virus, an acute clinical relapse of anaplasmosis was reported to occur in chronically infected cattle.[56] Failures in the premunition of cattle against babesiosis were reported to be due to the deleterious effects of starvation,

parturition and intercurrent disease, especially to infection with Anaplasma.[7]

The results of an experiment designed to study the clinical-hematological response of splenectomized calves simultaneously infected with A. marginale and B. bigemina has been reported.[52] The authors found that during the concurrent infection only one of the hemoparasites established a high parasitemia. The invasion of erythrocytes by the second hemoparasite, though initially substantial, decreased and occasionally ceased prematurely. The infected calves were divided into 2 groups: (1) calves with a high Babesia and a low Anaplasma parasitemia; and (2) calves with a high Anaplasma and a low Babesia parasitemia. The authors proposed that a non-antibody inhibiting factor was produced and allowed the hemoparasite with the highest established parasitemia to prevail while further invasion by the second hemoparasite was inhibited resulting in a subsequent decrease in the number of parasitized erythrocytes. When both the Anaplasma and the Babesia parasites established parasitemias of approximately equal levels, the parasites were mutually inhibited resulting in neither of the organisms establishing the expected level of parasitemia.

An experiment to study the development of babesiosis in calves which were in the convalescent stage of an Anaplasma infection has been conducted.[83] The author observed the Babesia organisms to have a prepatent period of 21 to 31 days, which was 16 to 21 days longer than the prepatent period usually observed in babesiosis.

The prolonged prepatent period was attributed to the high number of reticulocytes present in the blood during the recovery phase of anaplasmosis. The resistance of immature erythrocytes to invasion by Anaplasma and by Babesia parasites has been suggested in other reports.[52,85] A low Babesia parasitemia was also observed, especially in those cattle which had previously experienced a high Anaplasma parasitemia. The anemia observed in the experimentally infected calves was considered to have been due to the primary anaplasmosis, while the maintenance of the anemia was attributed to the secondary Babesia infection.

The experimental infection of cattle with Anaplasma or with Babesia parasites has been primarily accomplished by the transfer of blood from acute or chronically infected cattle to susceptible experimental animals. The procedure has required the maintenance of donor cattle. A more economical and convenient method for producing experimental infection was reported to be by the preparation of inocula which could be conveniently stored and used as needed.[3,4] The preservation and storage of Anaplasma and Babesia in blood and the subsequent use of blood as inocula to produce experimental infection has been reported by numerous investigators.[3,4,14,20,33,3 55,59,65,77,78,84] Various techniques were employed in the preparation, preservation and storage of infected blood to be used as inocula. Glycerol was used as a preservative or cryoprotectant in quantities ranging from 7 to 50% by volume for the preservation of Anaplasma[4,59,65,77,78] and for the preservation of Babesia.[4,20,59]

Dimethylsulfoxide was also used as a protectant in preservation procedures.[14,40,57,70] Following the addition of a preservative, preservation and storage of infected blood were accomplished by freezing and storage at low temperatures ranging from -70 to -195C. Anaplasma organisms stored in frozen bovine blood were reported to maintain virulence and pathogenicity for periods of 5 to 54 months.[4, 59,77,78] Babesia organisms were reported to suffer no loss in virulence following storage of infected frozen bovine blood for periods up to 24 months.[4,59]

CHAPTER III

MATERIALS AND METHODS

Experimental Organisms

Isolation

Pure isolates of A. marginale and of B. bigemina used during the experiment were obtained by natural Boophilus microplus transmission of the organisms to susceptible calves under field conditions. Two intact and 2 splenectomized 4 month old, Holstein-Friesian calves, with a known history of no exposure to tick vectors, negative complement fixation tests for Anaplasma and for Babesia and negative blood smears for hemoparasites were transported by airplane from the savanna of Bogotá to the North Coast of Colombia and placed in tick infested pastures at the Turipaná Instituto Colombiano Agropecuario (ICA) experimental station near Monteria, Cordoba. Daily blood samples were collected from the calves, and packed cell volumes and body temperatures were ascertained. Geimsa stained blood smears were examined daily for the presence of hemoparasites.

Babesia bigemina isolation. Following 18 days exposure to tick vectors, one of the splenectomized calves was observed to be infected with B. bigemina and B. argentina based on the examination of stained blood smears. One hundred ml. of blood was collected from the calf using 2 mg. of ethylenediaminetetraacetic acid (EDTA) disodium salt/ml. of blood as an anticoagulant, transported to Bogotá, and

immediately upon arrival inoculated intravenously into a splenectomized calf. The separation of B. bigemina from B. argentina and from A. marginale was accomplished by the rapid serial passage of infected blood through 5 splenectomized calves following a previously described method.[9] Blood was collected from the last calf of the rapid transfer series and inoculated into 2 intact, 6 month old Holstein-Friesian calves. The last 2 calves of the rapid transfer series were then treated intravenously with trypan blue[a] to prevent death from acute babesiosis resulting from infection with B. bigemina to allow the continued observation of the calves for infection with B. argentina. Packed cell volumes and Giemsa stained blood smears collected from the last 2 splenectomized calves of the rapid transfer series and from the 2 inoculated intact carrier calves were examined twice weekly for 12 weeks following inoculation to ascertain the purity of the B. bigemina isolate and the absence of other hemoparasites.

Anaplasma marginale isolation. A mixed infection of A. marginale, B. bigemina and B. argentina was observed in an intact calf at the ICA experimental station 32 days after exposure to tick vectors. One hundred ml. of blood was collected from the calf using EDTA disodium salt as an anticoagulant, transported to Bogotá and immediately upon arrival inoculated intravenously into a splenectomized calf. The splenectomized calf was treated intramuscularly at the time of inoculation and for 2 consecutive days thereafter with

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[a]Azul Tripan: Velox Laboratories, Bogotá, Colombia.

4-4', diamidino diazoaminobenzene diaceturate[b] at a dosage of 3.5 mg./kg. of body weight to eliminate the Babesia infection.[3] Packed cell volumes and Giemsa stained blood smears were examined from the calf 3 times weekly for 6 weeks following inoculation to observe the clinical progression of anaplasmosis and to ensure that the Babesia were eliminated by chemotherapy.

Preservation and Storage of Babesia bigemina

Inocula containing B. bigemina were prepared from the blood of the last splenectomized calf of the rapid transfer series, which had a pure patent infection. Immediately prior to preparation of the inocula, the calf was observed to have an erythrocyte count of 8.5×10^6 erythrocytes/mm.^{3} of blood with 2.5% of the erythrocytes being infected with B. bigemina. Each ml. of blood from the calf was calculated to contain approximately 2.1×10^8 B. bigemina. Five ml. of blood was aseptically collected from the calf into 7 ml. evacuated glass tubes[c] using 2 mg. of EDTA/ml. of blood as an anticoagulant. Following the collection of blood, sterile 95.8% glycerol[d] was added to each tube as a cryoprotectant to produce a final glycerol concentration of 10.7% by volume. The blood and glycerol were mixed by inverting the tubes several times and the tubes placed in a

[b] Ganaseg: E. R. Squibb & Sons, Interamerican Corporation, Cali, Colombia.

[c] B. D. Vacutainer: Becton-Dickenson and Company, Rutherford, N. J.

[d] J. T. Baker Chemical Company, Phillipsburg, N. J.

refrigerator at 4 C. Following 2 1/2 hours of refrigeration, the tubes were removed from the refrigerator; and the blood and glycerol mixture was frozen by plunging the tubes into a solid carbon dioxide --absolute alcohol bath. The tubes were immediately placed in a dry ice chest at -79 C. and stored until used as inocula.

Preservation and Storage of Anaplasma marginale.

Inocula containing A. marginale were prepared from the blood of a splenectomized calf having a pure patent infection. Immediately prior to preparation of the inocula, the calf was observed to have an erythrocyte count of 5.5×10^6 erythrocytes/mm.^{3} of blood with 3.5% of the erythrocytes being infected with A. marginale. Each ml. of blood from the calf was calculated to contain approximately 1.9×10^8 A. marginale. The inocula were prepared from the blood of the calf using a modification of a method previously reported.[4] Blood was aseptically withdrawn from the calf and collected in a 250 ml. sterile flask using 4 units of heparin/ml.[e] of blood as an anticoagulant. Modifications of this method included: the addition of sterile 95.8% glycerol to the blood to produce a final concentration by volume of 8.2% glycerol; five ml. aliquots of the blood and glycerol mixture were dispensed into 7 ml. glass tubes and the tubes sealed with rubber stoppers; temperature decrease during the freezing process was 1 C. per minute to -16 C., at which time the temperature

[e]Heparin (Ammonium salt): Distributed by Scientific Products Division, American Hospital Supply Corporation. General Offices Evanston, Illinois.

was lowered to -79 C. over a 5 minute period. The tubes of frozen blood were placed in a dry ice chest at -79 C. and stored until used as inocula.

Experimental Animals

Experimental Calves.

Twenty-two, male, nonsplenectomized, Holstein-Friesian calves, 7 months of age were used as experimental animals. Each calf was identified by an ear tag number and data collected during the experiment was referred to by these numbers. The calves were housed in a tick free environment for 2 months prior to and during the course of the experiment. Insect control was maintained by the periodic spraying of the housing facilities with an appropriate insecticide.[f] A balanced ration of ensilage and a concentrate mixture composed of 43% corn, 30% cotton seed oil meal, 24% bran, 1% salt, 1% bone meal and 1% trace minerals was provided to the calves during the experiment. Water and salt were provided free choice. Fecal specimens were collected from each calf prior to experimental infection and examined for helminth and protozoan parasites and treatment was deemed to be unnecessary.

Prior to experimental infection, all calves were screened for the presence of current or previous infection with Anaplasma or with

[f]Vapona: 0-0 dimetil 1-2-2 diclorovinil fosfate, Shell Ltda., Bogotá, Colombia.

Babesia parasites by the complement fixation test,[4,61] by the examination of stained blood smears and by the inoculation of a splenectomized calf with a pooled blood sample composed of 10 ml. of blood collected from each experimental calf. Blood samples were collected weekly from the splenectomized calf for 12 weeks following inoculation of the pooled blood sample, and packed cell volumes and stained blood smears were examined to ascertain the absence of infection.

The calves were examined physically, and blood and serum samples were collected twice weekly for 2 weeks prior to experimental infection to ascertain normal values for the following parameters: packed cell volume; hemoglobin concentration; total and differential leukocyte counts; reticulocyte count; total, direct, and indirect serum bilirubin concentration; serum glutamic-oxalacetic transaminase activity; serum sorbitol dehydrogenase activity; total serum protein content; serum protein electrophoretic patterns; body weights; and body temperature. Following the collection of blood and serum samples necessary to ascertain preinfection values for the above mentioned parameters, the experimental calves were randomly divided into 4 groups. Groups I and II each contained 3 calves, while Groups III and IV each contained 8 calves.

Inoculation of Experimental Calves.

Immediately prior to the inoculation of the experimental calves, the tubes of frozen infected blood were removed from the dry ice ches

and thawed by immersing the tubes in a water bath at 38 C. for 1 minute. The calves were inoculated within 30 minutes after thawing the frozen inocula.

The 3 calves of Group I were each subcutaneously injected with 5 ml. of thawed inoculum containing approximately 9.5×10^8 A. marginale. Group I served as a control to evaluate the response of calves singularly infected with A. marginale.

The 3 calves of Group II were each subcutaneously injected with 5 ml. of thawed inoculum containing approximately 10.5×10^8 B. bigemina. Group II served as a control to evaluate the response of calves singularly infected with B. bigemina.

The 8 calves of Group III were each subcutaneously injected with 5 ml. of thawed inoculum containing approximately 9.5×10^8 A. marginale. Seventy days following the inoculation of Group III, the calves had recovered from the acute clinical stage of anaplasmosis and were entering the carrier or post patent period as evidenced by a continual increase in packed cell volume and a continual decrease in the number of infected erythrocytes. The calves of Group III were each subcutaneously injected on day 70 with 5 ml. of thawed inoculum containing approximately 10.5×10^8 B. bigemina. Group III served to evaluate the effect of a secondary concurrent infection with B. bigemina in calves recently recovered from anaplasmosis.

The 8 calves of Group IV were each subcutaneously injected with 5 ml. of thawed inoculum containing approximately 10.5×10^8 B. bigemina. Thirty-seven days following the inoculation of Group IV,

the calves had recovered from the acute clinical stage of babesiosis as evidenced by a continual increase in packed cell volume and a continual decrease in the number of infected erythrocytes. The calves of Group IV were each subcutaneously injected on day 37 with 5 ml. of thawed inoculum containing approximately 9.5×10^8 A. marginale. Group IV served to evaluate the effect of a secondary concurrent infection with A. marginale in calves recently recovered from babesiosis.

Experimental Procedures

Sample Collection Schedule and Procedures.

Blood and serum samples were collected twice weekly from each calf for the duration of the investigation. This schedule was altered during the acute clinical phases of anaplasmosis and babesiosis during which time samples were collected 3 times weekly. Samples were collected from the calves of all 4 groups until day 120 post infection.

Samples were collected between 8:00 and 10:00 A.M. Body temperature, in degrees centigrade, was recorded during each collection period; and the general attitude and condition of each calf were noted. Blood and serum samples were aseptically collected from the calves by intravenous puncture of the jugular vein. A total of 45 ml. of blood was withdrawn from each calf at each collection. Five ml. of the blood was placed in a 10 ml. glass tube with 2 mg. disodium EDTA/ml. of blood as an anticoagulant. The remaining 40 ml. of blood

was dispensed into 10 ml. glass tubes. The blood was allowed to clot at 24 C. and the serum collected following centrifugation at 2500 r.p.m. for 15 minutes.

The body weight of each calf was approximated by tape measurement[g] made on 2 successive days at the beginning and at the termination of the experiment. Initial and final weights were obtained from the average values of the above mentioned measurements. Total weight gain from day 0 to 120 was calculated and average daily gain determined for each of the 4 groups of calves. An analysis of variance was conducted to determine if the differences observed in the mean initial weights, final weights, total gains and average daily gains between the 4 groups of calves was statistically significant.[76]

Bone marrow biopsies were taken from 2 calves randomly selected from each of the 4 experimental groups prior to experimental infection, and myeloid:erythroid ratios determined to approximate normal hematopoietic activity. Following experimental infection, bone marrow biopsies were again collected on days 50, 90 and 120 post infection. All bone marrow specimens were collected by biopsy from the medullary cavity of the rib using a previously described technique.[12]

Gross and Microscopic Pathology.

Pathological changes were evaluated by gross and by histopathological examinations. Four calves, 2 from Group III and 2 from

[g]Metric tape for milk breeds c3115, NASCO, Fort Atkinson, Wis.

Group IV, died during the experiment. A necropsy was performed on each of the calves and a complete set of tissue specimens were collected for histopathological examination. In addition, 2 concurrently infected calves from each of Groups III and IV were euthanatized by electrocution, using 110 volt alternating current, for necropsy on the day following maximum parasitemia of Babesia and Anaplasma respectively. Tissue specimens were collected from grossly normal and pathologic organs. The tissues were fixed in buffered 10% formalin for 24 hours, embedded in paraffin, sectioned at approximately 5 microns, stained with hematoxylin and eosin and examined by light microscopy. Special attention was given to the tissues of the reticuloendothelial system during histopathological examination; however, tissues from all body systems were examined. A normal, non-infected control calf was also euthanatized in the same manner and subjected to necropsy. A complete set of tissue specimens were similarly collected, processed and utilized for comparative studies.

Clinical Pathology.

The techniques performed on blood samples were completed on the same day as sample collection. Techniques analyzing serum samples were performed on samples which had been frozen and stored at -20 C.

Packed cell volumes were determined by the microhematocrit method.[68] An analysis of variance was conducted to determine if the differences observed in the mean packed cell volumes between the

significant.[6]

Hemoglobin concentration was measured by the Hycel cyanmethemoglobin technique.[h] Differential and total leukocyte counts were determined by standard laboratory techniques.[6] Reticulocyte counts were conducted using a standard reported technique, utilizing brilliant cresyl blue stain followed by counter staining with Wright's stain.[68]

The percentage of parasitized erythrocytes was determined by the microscopic examination of thin blood smears under the oil immersion objective. Following fixation in absolute methanol, the blood smears were stained for 20 minutes with a 1 to 20 solution of Giemsa, using phosphate buffered distilled water at a PH of 7.0 to 7.1 to prepare the stain solution. A wetting agent, alkyl phenoxy polyethoxy ethanol[i] (APPE), was added to the buffered distilled water to a final concentration of 0.01% to aid in the removal of precipitated stain from the blood smears. Stock solutions of M/15 Na_2HPO_4, M/15 NaH_2PO_4, and 10% APPE were prepared and stored in separate bottles. Fresh buffered water for making the stain solution was prepared as needed by using 39 ml. of M/15 NaH_2PO_4, 61 ml. of M/15 Na_2HPO_4, 1 ml. of 10% APPE and 899 ml. of distilled water. The stained blood smears were washed in distilled water and dried under a heat lamp. The percentage of parasitemia was determined by counting from 10,000 to

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[h] Hycel, Houston, Texas

[i] Triton X-100, Rohm and Haas, Philadelphia, Penn.

12,000 erythrocytes on each slide. All counts were made by counting cells located from 1/4 to 1/2 inch from the feathered end of the blood smear. Thick blood smears were used for the detection of Babesia parasites in the blood of chronically infected calves.[42] An analysis of variance was conducted to determine if the differences observed in the mean prepatent periods and in the mean parasitemias between the calves singularly and concurrently infected was statistically significant.[76]

Total serum protein levels were ascertained by using a handheld, temperature compensated refractometer.[5j] Serum glutamic oxalacetic transaminase levels were measured by a kit procedure[k] modified from the original method.[60] Serum sorbitol dehydrogenase activity was measured by a modified kit procedure[m] adopted from an original method.[21] Serum bilirubin concentration was determined by a modification of the Van den Bergh procedure.[22]

Serum protein electrophoretic patterns were ascertained by a modification of a technique using cellulose acetate membranes.[8] Five lambdas of serum were applied to cellulose acetate membranes[n] with a Titan M-2 applicator.[o] The cellulose membranes were presoaked in cold trisbarbitol-sodium barbitol buffer[p] having a PH of 8.8 and an

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[j] T-S Meter, American Optical Company, Buffalo, N. Y.

[k] Sigma Chemical Company, St. Louis, Mo.

[m] Sigma Chemical Company, St. Louis, Mo.

[n] Sepraphore III: Gelman Instrument Company, Ann Arbor, Mich.

[o] Helena Laboratories, Allen Park, Miss.

[p] High Resolution Buffer, 51104, Gelman Instrument Company, Ann Arbor, Mich.

ionic strength of 0.045. Following application of the serum samples, the membranes were immediately transferred to an electrophoresis chamber.[q] Separation of the serum protein fractions was accomplished by applying a constant voltage of 300 volts or a constant amperage of 1.25 milliamperes per membrane to the chamber for 45 minutes. Following electrophoretic separation, the membranes were immediately placed in a solution of 0.5% Ponceau[r] with 100 ml. of trichloroacetic acid and fixed-stained for 5 minutes. Excess and background stain was removed by rinsing the membranes for 1 minute in each of four 5% acetic acid baths. The membranes were then dehydrated by immersion for 30 to 60 seconds in absolute methyl alcohol and cleared by immersion in a solution of 10% acetic acid in methyl alcohol. Following 20 minutes of drying at 55 C., the membranes became transparent. The various serum protein fractions on the transparent membranes were quantitated by using a densitometer with an automatic scanner and intergrator.[s] Absolute values for the various protein fractions were obtained from the relative percentages of each fraction and the total serum protein values.

Serology.

Serum samples collected from each experimental calf were screened

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[q]Deluxe Electrophoresis Chamber 51170, Gelman Instrument Company, Ann Arbor, Mich.

[r]Gelman Instrument Company, Ann Arbor, Mich.

[s]Gelman Automatic Recording and Intergrating Scanner, No# 39372, Gelman Instrument Company, Ann Arbor, Mich.

for the presence of complement fixing antibodies for A. marginale and for B. bigemina. All samples were screened for the presence of A. marginale antibody by the standardized complement fixation test for anaplasmosis as prescribed by the United States Department of Agriculture.[2] The complement fixation test employed to detect B. bigemina antibodies was a previously described modification[80] of the original test.[41] All serum samples found to be positive by these screening procedures were then titrated by a microprocedure technique.[24] Antigen used in the complement fixation test for anaplasmosis was obtained from the United States Department of Agriculture.[t] Antigen used in the complement fixation test for babesiosis was obtained by a described method using a distilled water extract of the parasite suspension.[39] Antigen thus obtained was stored at -79 C. until used in the complement fixation test.

[t]Animal Disease Eradication Branch, Agricultural Research Service, U. S. D. A.

CHAPTER IV

RESULTS

Experimental Organisms

Isolation of Babesia bigemina.

The first of the series of 5 splenectomized calves used in the isolation of B. bigemina by the rapid serial passage of infected blood was inoculated intravenously with 100 ml. of blood collected from a splenectomized calf naturally infected by B. microplus at the Turipaná ICA station. The naturally infected calf was observed to be infected with B. bigemina, B. argentina and A. marginale. The prepatent periods for the 3 hemotropic parasites in the 5 splenectomized calves are given in Table 1. Babesia argentina was not observed in blood smears from either the fourth or the fifth splenectomized calves. Anaplasma marginale was not observed in blood smears from either the second, third, fourth or fifth splenectomized calves. Inocula containing pure B. bigemina were prepared from the infected blood of the fifth splenectomized calf of the rapid transfer series.

Isolation of Anaplasma marginale.

The splenectomized calf used for the chemotherapeutic separation of A. marginale was inoculated with 100 ml. of blood collected from a naturally infected intact calf at the Turipaná ICA station. The

TABLE 1. The Separation of Babesia bigemina from Babesia argentina and Anaplasma marginale by the Rapid Serial Passage of Infected Blood through 5 Splenectomized Calves.

Splenectomized Calf No.	Prepatent Period (Days)		
	Babesia bigemina	Babesia argentina	Anaplasma marginale
1	1.50	4.00	44.00
2	1.25	4.00	Neg.
3	1.00	15.00	Neg.
4	1.50	*Neg.	Neg.
5	2.50	Neg.	Neg.

*Neg. -- No parasites seen in Giemsa stained blood smears.

naturally infected calf was observed from stained blood smears to be infected with A. marginale, B. bigemina and B. argentina. Erythrocytes infected with A. marginale were seen in the blood of the splenectomized calf on day 12 PI. The number of infected erythrocytes gradually increased to 5% on day 23 PI and then gradually decreased. Babesia sp. were not observed in blood smears from the splenectomized calf. Inocula containing pure A. marginale were prepared from the infected blood of the splenectomized calf on day 15 PI when the number of infected erythrocytes was observed to be approximately 3.5%. The splenectomized calf died on day 42 PI in a state of severe anemia.

Experimental Procedures

Clinical Observations.

Slight elevations of rectal temperatures were observed in the 4 groups of calves. An average maximum elevation of rectal temperature of 1.5 C. was observed in the calves of Group I, singularly infected with A. marginale, and in the calves of Groups III and IV, concurrently infected with A. marginale and B. bigemina. The slight elevation of temperature occurred during the period when Anaplasma marginal bodies were first observed in the erythrocytes of the infected calves. The temperature of the calves returned to normal within 10 to 15 days and remained normal for the remainder of the experiment. Elevation of rectal temperature was not observed in the calves following infection with B. bigemina. Calf #134 of Group III was the only exception to this observation. The calf was in the recovery phase of anaplasmosis when it was inoculated on day 70 PI with B. bigemina. On day 78 PI, the calf had a rectal temperature of 40.8 C. and hemoglobinuria was observed. The calf died on day 79 PI.

The general physical condition of the calves of Groups I, III and IV declined as indicated by poor body condition, dull haircoats and inactivity. The calves of Group II singularly infected with B. bigemina remained active and in good physical condition throughout the experiment.

Body weight increases and average daily gains were calculated by finding the difference between initial weights on day 0 and the

final weights on day 120 PI. Total weight gain, and average daily gain was approximately the same in the calves of Groups I, III and IV infected with A. marginale (Table 2). The calves of Group II, infected with B. bigemina, were observed to have an average daily gain and a total weight gain twice that of the other 3 groups. The differences observed in the mean total weight gains and in the average daily gains between the calves of Group II and the calves of Groups I, III and IV were statistically significant at the 0.01 level of significance.

Gross Pathology.

Calf #134 of Group III, which was in the recovery phase of anaplasmosis, died on day 79, 9 days after being inoculated with blood containing B. bigemina. Gross lesions included: hepatomegaly with yellow discoloration; splenomegaly and congestion; petechial hemorrhages in the renal capsule, pericardium, prefemoral and renal lymph nodes; distention of the urinary bladder with port wine colored urine; distention of the gall bladder with thick yellow bile; serosanguineous fluid in the pericardium; congested kidneys; dilatation of the right ventricle of the heart; paleness of oral and conjunctival mucous membranes; and general enlargement of the body lumph nodes with prominent lymphoid follicles on the cut surfaces.

Two calves from each of Groups III and IV and a normal noninfected control calf were euthanatized and subjected to necropsy. Gross lesions of the infected calves included: 500 to 1000 ml. of yellow

TABLE 2. Initial and Final Weights with Total Weight Gains and Average Daily Gains of the 4 Groups of Calves Experimentally Infected with Anaplasma marginale and/or Babesia bigemina. Group Means and Standard Deviations from the Mean are Presented.

GROUPS	Number of Calves Per Group	Initial Weight (kg.) Day 0 Mean±S.D.*	Final Weight (kg.) Day 120 Mean±S.D.	Total Gain (kg.) Mean±S.D.	Average Daily Gain (kg.) Mean±S.D.
I	3	135±9	182±6	48±12	0.40±0.11
II	3	145±20	240±35	95±15	0.79±0.11
III	8	144±21	183±30	39±10	0.32±0.09
IV	8	133±8	176±25	43±21	0.35±0.18
Significance:		NS**	P<0.05	P<0.01	P<0.01
DRS†			63	37	0.30

*Standard Deviation from the Mean

**Not Significant

†Difference Required for Significance

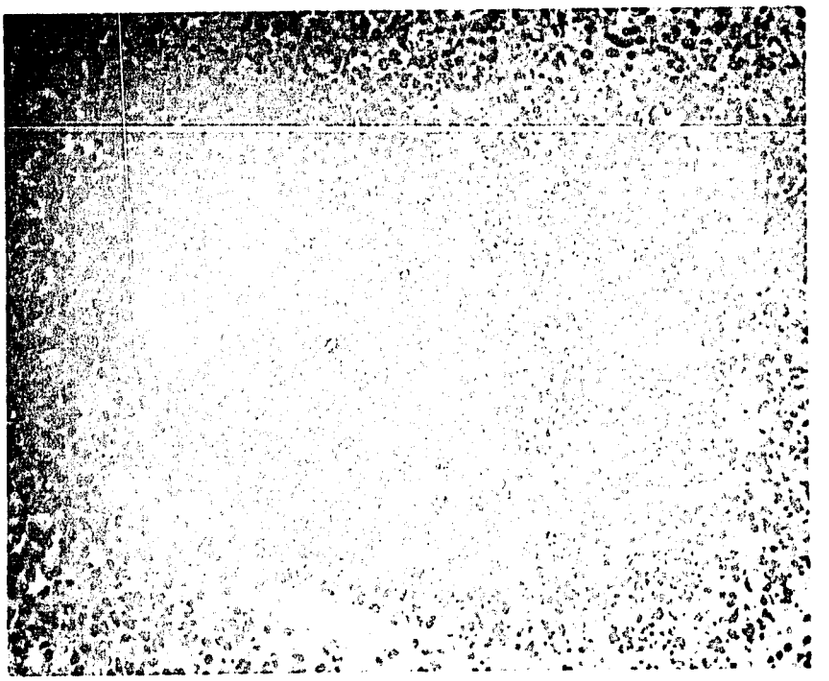
fluid in the peritoneal cavity, 100 to 200 ml. of fluid in the pleural cavity, and occasionally fluid in the pericardial sac; moderate lymph node enlargement; splenomegaly with prominent splenic follicles; renal congestion; moderate hepatomegaly; and occasional serous atrophy of depot fat. No differences in the extent or severity of gross lesions were observed between the 2 calves of Group III and the 2 calves of Group IV. An additional calf from Group III and 2 calves from Group IV died during the experiment. Gross and histopathological examinations indicated 2 of the calves died from pneumonia and the other from a strangulated ventral abdominal hernia.

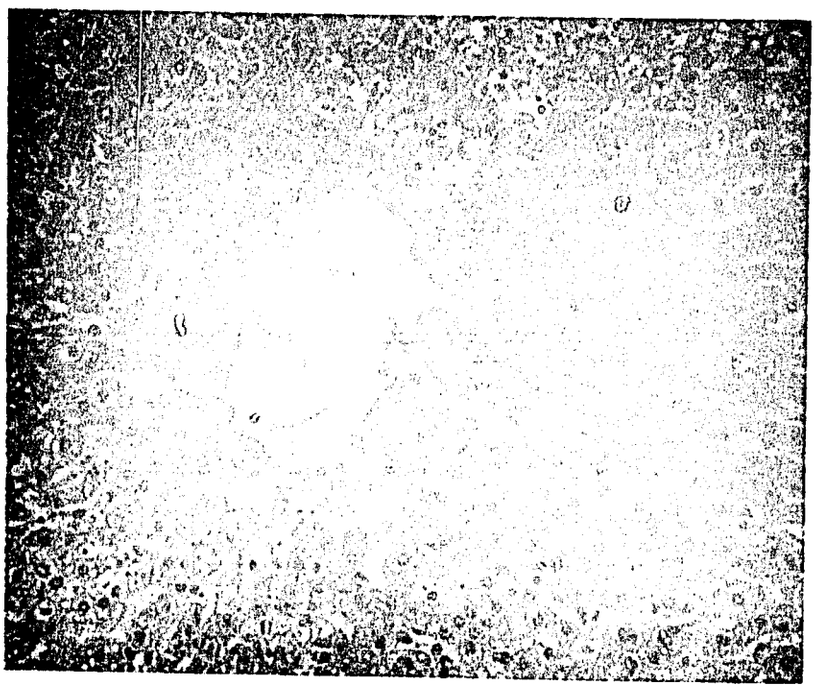
Microscopic Pathology.

The spleen of calf #134, from Group III, which died during the experiment, was severely congested and had focal areas of necrosis in the lymphoid follicles (Fig. 1). Granules of hemosiderin were observed in reticuloendothelial cells in the spleen, glomerular tufts, Kupffer cells and within alveolar septal macrophages in the lungs. The liver was moderately congested and degenerative and necrotic changes were observed in numerous individual hepatocytes in the centrolobular areas (Fig. 2). Kupffer cell hypertrophy and erythrophagocytosis were prominent. Emphysema was observed in most of the pulmonary lobules, and a moderate infiltration of lymphocytes and reticuloendothelial cells was observed in the peribronchial areas. The basement membranes of the glomerular tufts were relatively thickened. Casts of hemoglobin were observed in the lumens of several renal

Fig. 1. Spleen of calf #134 experimentally infected with Anaplasma marginale on day 0 and with Babesia bigemina on day 70 of the experiment. Congestion and focal areas of necrosis in the lymphoid follicles was observed. H&E. 560X.

Fig. 2. Liver of calf #134 experimentally infected with Anaplasma marginale on day 0 and with Babesia bigemina on day 70 of the experiment. The liver was moderately congested and degenerative and necrotic changes were observed in numerous individual hepatocytes in the centrolobular areas. H&E. 560X.





tubules. Necrosis of individual lymphocytes was observed in the lymphoid follicles of the lymph nodes. Numerous nucleated erythrocytes were observed in the sinuses of the lymph nodes and spleen and in the sinusoids of the liver.

Tissue sections from each of the 2 calves of Group III and the 2 calves of Group IV euthanatized for dissection were grouped together by organ and examined for microscopic abnormalities. No differences were observed in the extent or severity of histologic lesions between the calves of Group III and Group IV.

Liver. Slight to moderate centrolobular degeneration of the hepatocytes was present, as evidenced by numerous hepatocytes having pyknotic and karyorrhectic nuclei and eosinophilic cytoplasm. A slight to moderate infiltration of lymphocytes and reticuloendothelial cells was observed in the periportal areas. The hepatic sinusoids contained an excessive number of mononuclear inflammatory cells and immature nucleated erythrocytes. Kupffer cell hypertrophy was observed in most of the sections. Erythrophagocytosis and phagocytized hemosiderin granules were occasionally observed.

Kidney. An eosinophilic proteinaceous material was observed in Bowman's capsules and in the lumens of the renal tubules. A slight infiltration of lymphocytes and plasma cells was occasionally observed in the interstitial tissues and was predominantly perivascular in distribution. Phagocytized hemosiderin granules were occasionally seen in macrophages in the glomerular tufts and in the interstitium.

Spleen. A moderate to marked lymphoid hyperplasia of the malpighian corpuscles was commonly observed. Hyperplasia of reticuloendothelial cells in the red pulp was observed in some sections. Granules of phagocytized hemosiderin were observed in the sinuses of all sections.

Lymph nodes. Histopathological examinations were conducted on tissue sections prepared from the suprapharyngeal, prescapular, prefemoral, sternal, bronchial, hepatic, renal and mesenteric lymph nodes. The microscopic abnormalities observed were similar irregardless of which lymph node was examined. Moderate hyperplasia of the lymphoid follicles was observed in all sections, with marked hyperplasia being observed in several sections. Hyperplasia of reticuloendothelial cells was occasionally observed. Granules of phagocytized hemosiderin were present in several sections.

Lung. Microscopic abnormalities were seldom observed and consisted of occasional perivascular and interstitial infiltration of a few lymphocytes.

Cerebrum. Microscopic abnormalities were irregularly observed and consisted of occasional perivascular infiltration of small numbers of lymphocytes and plasma cells.

Adrenal and thyroid glands. No microscopic abnormalities were observed.

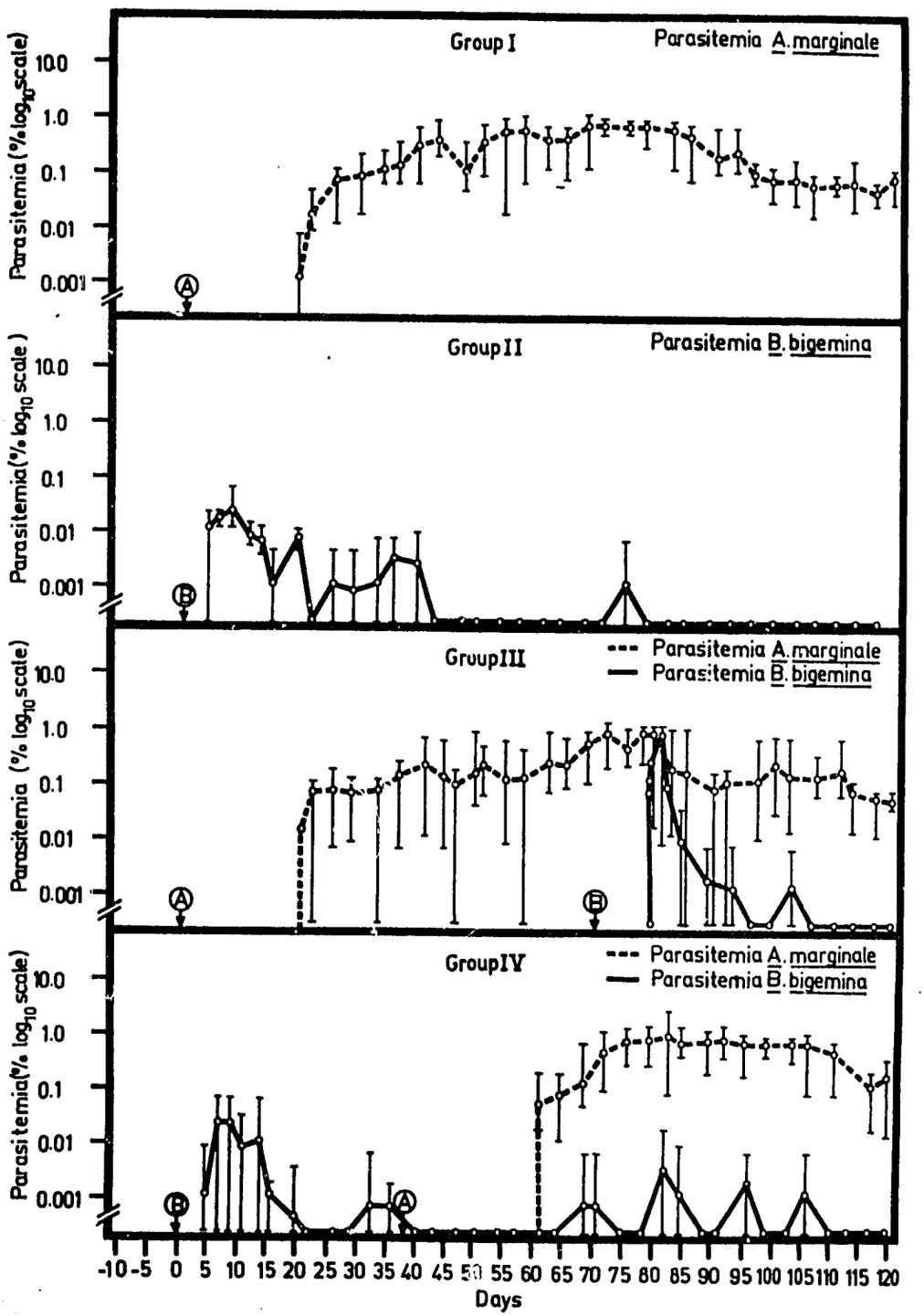
Clinical Pathology.

Prepatent periods and parasitemias. The 3 calves of Group I, which were inoculated with blood infected with A. marginale, were observed to have an average prepatent period of 21 days, with a range of 20 to 26 days. There was a fluctuating increase in the number of infected erythrocytes until an average maximum parasitemia of approximately 1.4% was observed on day 70 PI (Fig. 3). A continual decrease in the number of infected erythrocytes was observed from day 70 PI to day 120 PI at which time an average of 0.10% was observed to be infected.

The average prepatent period of the 3 calves of Group II, which were inoculated with blood containing B. bigemina, was 7 days, with a range of 5 to 9 days. An average maximum of 0.06% of the erythrocytes was observed to be infected on day 9 PI (Fig. 3). There was a fluctuating decrease in the number of infected erythrocytes until day 40 PI after which fewer than an average of 0.005% of the erythrocytes were observed to be infected.

The 8 calves of Group III, which were inoculated with blood containing A. marginale on day 0, were observed to have an average prepatent period of 21.7 days, with a range of 20 to 26 days. The number of erythrocytes infected with A. marginale increased until day 71 PI when an average maximum parasitemia of 1.1% was observed (Fig. 3). Following day 80 PI, a continual decrease in the number of infected erythrocytes was observed until day 120 PI when a 0.09% average was

Fig. 3. The percentages of parasitized erythrocytes of the 4 groups of calves experimentally infected with Anaplasma marginale and/or Babesia bigemina. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale (A), or with Babesia bigemina (B).


Group I
Parasitemia A. marginale
Group II
Parasitemia B. bigemina
GroupIII
Parasitemia A. marginale
Parasitemia B. bigemina
GroupIV
Parasitemia A. marginale
Parasitemia B. bigemina
Parasitemia (% log10 scale)
10.0
1.0
0.1
0.01
0.001
A
B
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
Days

infected. On day 70 PI, the calves of Group III were inoculated with blood containing B. bigemina. Erythrocytes infected with B. bigemina were observed 8 days later in each calf of the group. An average maximum parasitemia of 1.0% was observed on day 10 PI with B. bigemina, or on day 80 of the experiment. The number of erythrocytes infected with B. bigemina decreased until day 85 of the experiment (day 15 PI with B. bigemina), after which fewer than an average of 0.005% were infected.

The 8 calves of Group IV, which were inoculated with blood containing B. bigemina on day 0, were observed to have an average prepatent period of 7.5 days, with a range of 5 to 9 days. An average maximum parasitemia of 0.06% was observed on day 8 PI (Fig. 3). The number of erythrocytes infected with B. bigemina decreased until day 16 PI, after which fewer than an average of 0.006% was observed to be infected. On day 37 PI the calves of Group IV were inoculated with blood containing A. marginale. An average prepatent period of 25 days, with a range of 24 to 27 days, was observed. The number of erythrocytes infected with A. marginale increased until day 45 PI with A. marginale or day 82 of the experiment, when an average of 2.2% of the erythrocytes were infected. Following day 82, the number of Anaplasma infected erythrocytes decreased until an average of 0.40% was observed to be parasitized on day 120.

The difference of approximately 4 days observed in the average prepatent period of A. marginale in the calves singularly and secondarily infected was statistically significant at the 0.01 level of

significance. The difference of 1 day observed in the average prepatent period of B. bigemina in the singularly and secondarily infected calves was not statistically significant.

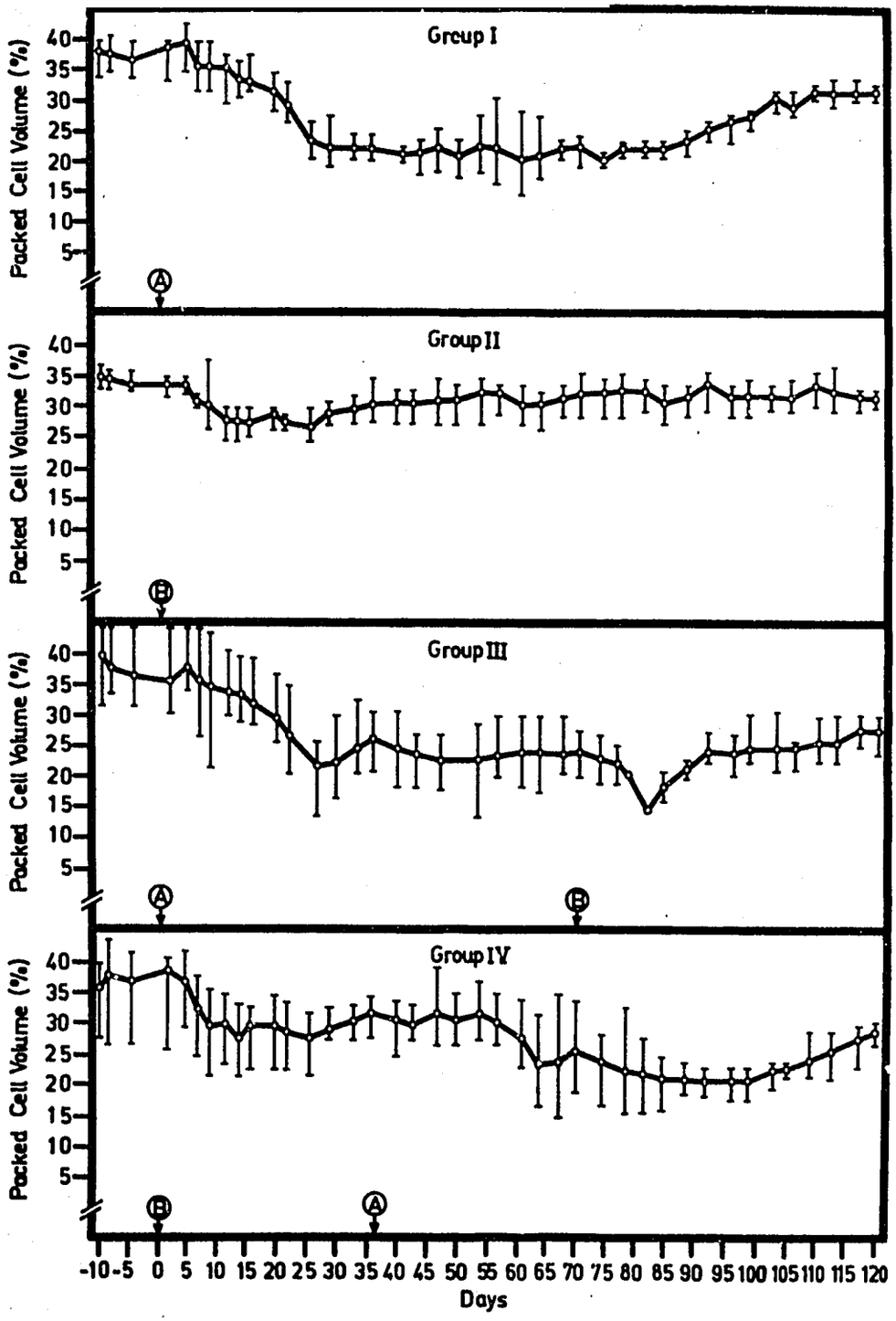
The difference of approximately 0.8% observed in the mean maximum number of erythrocytes parasitized by A. marginale in the singularly and secondarily infected calves was statistically significant at the 0.05 level of significance. The difference of approximately 0.94% in the maximum number of erythrocytes parasitized by B. bigemina in the singularly and secondarily infected calves was also statistically significant at the 0.05 level of significance.

Packed cell volume. A continual decrease in the average packed cell volume (PCV) of Group I was observed starting on day 20 PI with A. marginale and continuing to day 29 PI at which time the PCV was 22% with a range of 19 to 27% (Fig. 4). The PCV then fluctuated between 20 and 22% until day 90 PI, at which time a gradual increase occurred and continued until day 120 PI to a final level of 31%.

An average low PCV of 27% with a range of 25 to 29% was observed in Group II on day 16 PI (Fig. 4). The PCV increased to 30% by day 36 PI and remained above 30% for the remainder of the experiment.

The average PCV of the calves in Group III decreased to 21% with a range of 13 to 25% by day 26 PI with A. marginale. The PCV fluctuated between 21 and 24% until day 70 PI on which day Group III was inoculated with blood containing B. bigemina (Fig. 4). Following day 70 PI the PCV decreased until a low of 13% with a range of 8 to 19%

Fig. 4. Fluctuations in the packed cell volumes of the 4 groups of calves experimentally infected with Anaplasma marginale and/or Babesia bigemina. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale (A) or with Babesia bigemina (B).


Group I
Group II
Group III
Group IV
Packed Cell Volume (%)
40
35
30
25
20
15
10
5
A
B
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
Days

was observed on day 82 PI. A continual increase occurred from day 83 PI until day 120 PI at which time the average PCV for Group III had returned to 21%.

The average PCV of the calves of Group IV decreased to 27% with a range of 21 to 32% by day 14 PI with B. bigemina (Fig. 4). The PCV then increased to 31% on day 36 PI, and on the following day the calves of Group IV were inoculated with blood containing A. marginale. The average PCV decreased to 20% with a range of 18 to 23% by day 89 PI and fluctuated between 20 and 22% until day 106 PI at which time the PCV gradually increased to 28% by day 120 PI.

The difference observed between the minimum average PCV of the calves singularly infected with A. marginale and the calves secondarily infected with A. marginale following recovery from babesiosis was not statistically significant. The difference observed in the minimum average PCV of the calves singularly infected with B. bigemina and the calves secondarily infected with B. bigemina following recovery from anaplasmosis was statistically significant at the 0.01 level of significance.

Hemoglobin level. A continual decrease in hemoglobin concentration was observed in Group I until day 40 PI with A. marginale when an average low value of 6.8 gms.% with a range of 6.3 to 7.0 gms.% was observed. The hemoglobin concentration remained relatively stable, fluctuating between 6.7 and 7.2 gms.% from day 41 to day 82 PI when an increase in concentration occurred and continued to a level of 10.2 gms.% on day 120 PI.

An average low hemoglobin concentration of 8.6 gms.% with a range of 7.7 to 9.3 gms.% was observed in the calves of Group II on day 14 PI with B. bigemina. It returned to 10.0 gms.% by day 40 PI and remained stable until day 120 PI.

A continual decrease was observed in the average hemoglobin concentration of Group III starting on day 16 PI with A. marginale and decreasing to a low value of 6.5 gms.% with a range of 4.0 to 10.0 gms.% by day 26 PI. The hemoglobin level remained stable until day 70, at which time the calves in Group III were inoculated with blood containing B. bigemina. A decrease in hemoglobin concentration began on day 75 and continued until day 82 when an average low concentration of 4.4 gms.% with a range of 3.0 to 6.7 gms.% was observed. An increase was observed on day 89 and continued until day 120 PI when the hemoglobin concentration was observed to be 8.8 gms.%.

The calves of Group IV had an average hemoglobin concentration of 9.2 gms.% with a range of 6.7 to 10.7 gms.% on day 14 PI with B. bigemina. The hemoglobin concentration began to increase on day 20 PI and had returned to a concentration of 9.8 gms.% by day 36 PI. On day 37 PI the calves of Group IV were infected with A. marginale. The hemoglobin concentration decreased to 6.4 gms.% with a range of 5.7 to 8.3 gms.% by day 89 PI. A gradual increase began on day 90 PI and continued until day 120 PI when a final concentration of 8.9 gms.% was observed.

Total and differential leukocyte counts. A decrease in the total number of leukocytes was observed in the calves of Group I on

day 7 PI (Fig. 5). By day 12 PI, the total leukocyte count had returned to preinoculation values and remained stable with slight fluctuations thereafter. A moderate decrease in the number of lymphocytes and a slight decrease in neutrophils was observed in the calves of Group I on day 7 PI (Fig. 5). The number of monocytes increased to an average high of 1,900/mm.^{3} on day 20 PI and remained above preinoculation values thereafter (Fig. 6). The number of eosinophils increased until day 14 PI in the calves of Group I after which the number decreased to below preinfection levels (Fig. 6). The number of basophils remained stable in all Groups for the duration of the experiment.

A decrease in the total number of leukocytes was observed in the calves of Group II on days 7 to 12 PI (Fig. 5). The total leukocyte count of the calves of Group II returned to preinoculation values on day 14 PI and increased gradually until day 60 PI after which it fluctuated between 12,000 and 15,000/mm.^{3} for the remainder of the experiment. The number of lymphocytes and neutrophils decreased on days 7 to 10 and then returned to preinfection values with moderate fluctuations thereafter (Fig. 5). A monocytosis was observed in the calves of Group II starting on day 33 PI and continuing for the remainder of the experiment (Fig. 6). The number of eosinophils fluctuated in the calves of Group II with no remarkable changes being observed (Fig. 6).

A slight decrease in the total number of leukocytes was observed in the calves of Group III on days 7 to 9 PI (Fig. 5). The number of

Fig. 5. Hematological data. Total numbers of leukocytes, lymphocytes and neutrophils of the 4 groups of calves experimentally infected with Anaplasma marginale and/or Babesia bigemina. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale (A) or with Babesia bigemina (B).

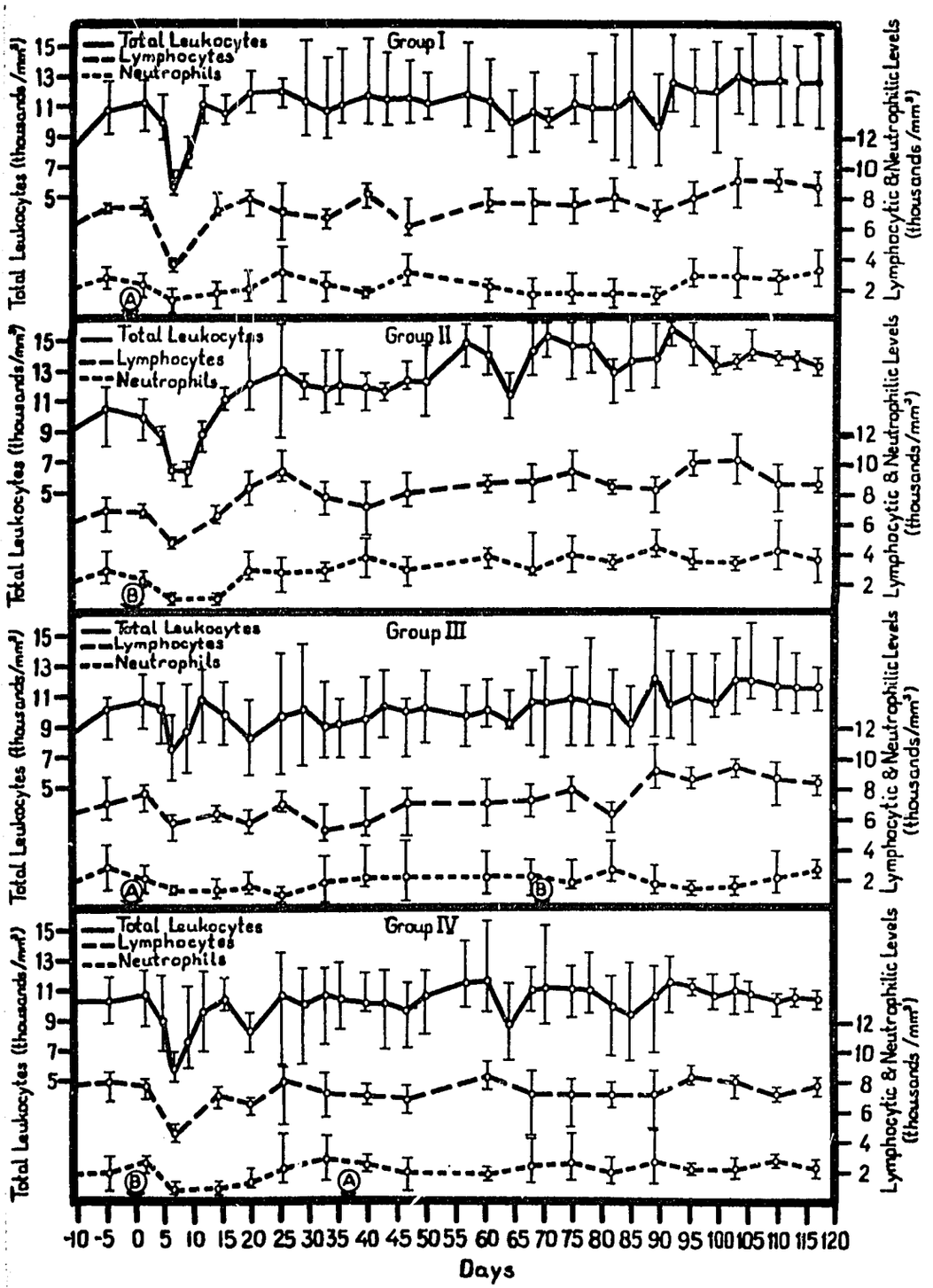
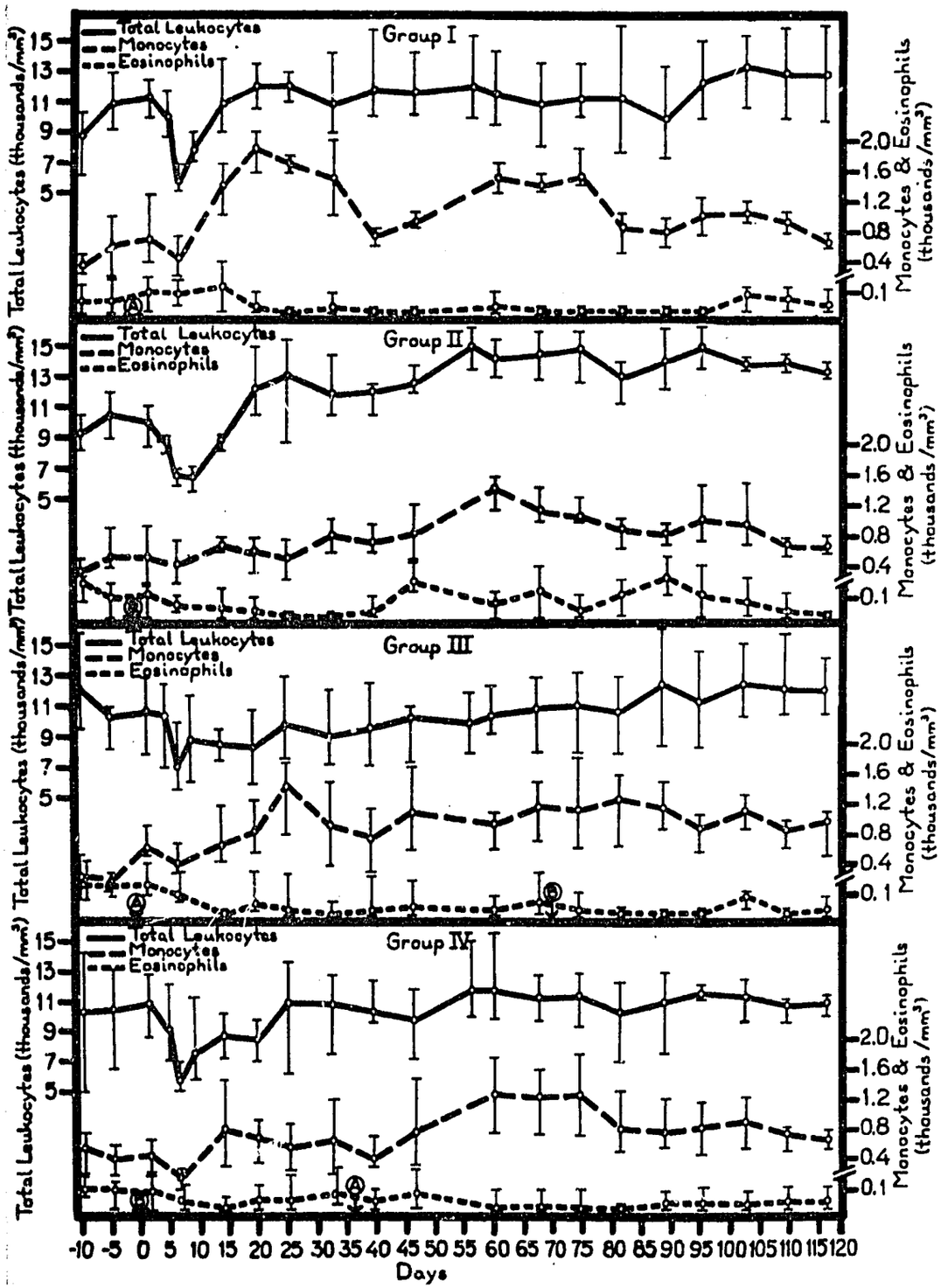

Group I
Group II
Group III
Group IV
Total Leukocytes
Lymphocytes
Neutrophils
Total Leukocytes (thousands/mm³)
Lymphocytic & Neutrophilic Levels (thousands/mm³)
A
B
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
Days

Fig. 6. Hematological data. Total numbers of leukocytes, monocytes and eosinophils of the 4 groups of calves experimentally infected with Anaplasma marginale and/or Babesia bigemina. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale (A) or with Babesia bigemina (B).


Group I
Group II
Group III
Group IV
Total Leukocytes
Monocytes
Eosinophils
Total Leukocytes (thousands/mm³)
Monocytes & Eosinophils (thousands/mm³)
15
13
11
9
7
5
2.0
1.6
1.2
0.8
0.4
0.1
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
Days

lymphocytes and neutrophils fluctuated near preinfection values for the duration of the experiment (Fig. 5). Eosinophils decreased following inoculation and remained below normal for the duration of the experiment (Fig. 6). The number of monocytes increased above preinfection values on day 14 PI and remained elevated thereafter (Fig. 6).

The total number of leukocytes decreased to 5,700/mm.^{3} in the calves of Group IV on day 7 PI (Fig. 5). It returned to preinfection values by day 16 PI and remained stable thereafter. The number of lymphocytes and neutrophils decreased briefly on days 7 to 14 of the experiment after which only slight fluctuations were observed (Fig. 5). The number of monocytes increased in the calves of Group IV on days 14 to 34 PI and returned to normal on day 40 PI (Fig. 6). Following day 40 PI, a monocytosis was observed for the remainder of the experiment. The number of eosinophils fluctuated near preinfection values in the calves of Group IV for the duration of the experiment (Fig. 6).

Reticulocyte count. Large, polychromatophilic erythrocytes were constantly observed in blood smears in each group of calves following day 25 of the experiment. The number of polychromatophilic erythrocytes was higher in those calves with lower packed cell volumes. Reticulocyte counts indicated that immature erythrocytes were present in Groups I and IV as early as day 14 PI and in Groups II and III on days 22 and 20 PI respectively (Table 3). The large polychromatophilic erythrocytes were not observed to be infected with Anaplasma

TABLE 3. Reticulocyte Counts of the 4 Groups of Calves Experimentally Infected with Anaplasma marginale and/or Babesia bigemina.

Days PI	GROUPS* I %	II %	III %	IV %
-4	NS**	NS	NS	NS
2	NS	NS	NS	NS
5	NS	NS	NS	NS
9	NS	NS	NS	NS
12	NS	NS	NS	NS
14	0.1	NS	NS	0.1
16	NS	NS	NS	NS
20	NS	NS	0.1	0.4
22	0.3	0.7	0.8	0.9
26	0.2	0.8	0.2	0.4
29	0.1	0.1***	0.2	1.8
33	0.3	ND	ND	ND
36	0.5	1.2	2.9	3.3
40	ND	ND	ND	ND
43	0.3	0.3	0.2	0.3
47	0.3	0.3	0.2	0.3
50	0.4	0.8	0.7	0.4
54	ND	ND	ND	ND
61	0.2	0.2	0.2	0.1
64	0.2	0.1	0.2	0.2
68	0.2	0.3	0.5	0.4
71	0.3	0.2	0.2	0.4
75	0.6	0.6	0.8	0.3
82	0.3	0.5	0.3	0.4
85	0.8	0.4	0.4	0.4
89	0.3	0.3	0.5	0.3
92	0.3	0.1	0.1	0.3
96	0.2	0.1	0.1	0.3
99	0.1	0.1	0.1	0.4
103	0.1	0.1	0.1	0.3
110	0.1	0.1	0.1	0.2
117	0.1	0.1	0.1	0.2

*Group means are presented

**NS -- Not seen

***ND -- Not determined

nor with Babesia parasites during any period of the experiment. The reticulocyte count increased to an average high of 0.5%, 1.2%, 2.9% and 3.3% for the 4 respective groups on day 36 PI. Following day 36 PI, the percentage of reticulocytes steadily decreased until the termination of the experiment on day 120.

Total serum proteins. An increase in the total serum protein (TSP) content was observed in Groups I, II and III on day 50 PI and in Group IV on day 71 PI (Fig. 7). The concentration of TSP remained substantially above preinoculation values in each of the 4 experimental groups for the remainder of the experiment.

Serum glutamic oxalacetic transaminase. A substantial increase above average preinoculation values was observed in the level of serum glutamic oxalacetic transaminase (SGOT) in the calves of Groups I and III on days 22 and 26 PI with A. marginale (Fig. 8). The average SGOT levels during this period were observed to be from 147 to 165 and 179 to 181 Frankel units for Groups I and III respectively. The SGOT concentration increased only slightly above preinoculation values in Group II from day 5 to 12 PI and then decreased to preinoculation values for the remainder of the experiment. The SGOT concentration increased in the calves of Group IV on days 5 to 9 PI with B. bigemina and again on days 60 to 65 which was equivalent to days 23 to 28 PI with A. marginale (Fig. 8).

Sorbitol dehydrogenase. An increase in average serum sorbitol dehydrogenase levels was observed in the calves of Group I on day 110 PI at which time a serum level of 1154 Sigma units/ml. with a

Fig. 7. Total serum proteins concentrations and albumin:globulin ratios of the 4 groups of calves experimentally infected with Anaplasma marginale and/or Babesia bigemina. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale (A) or with Babesia bigemina (B).

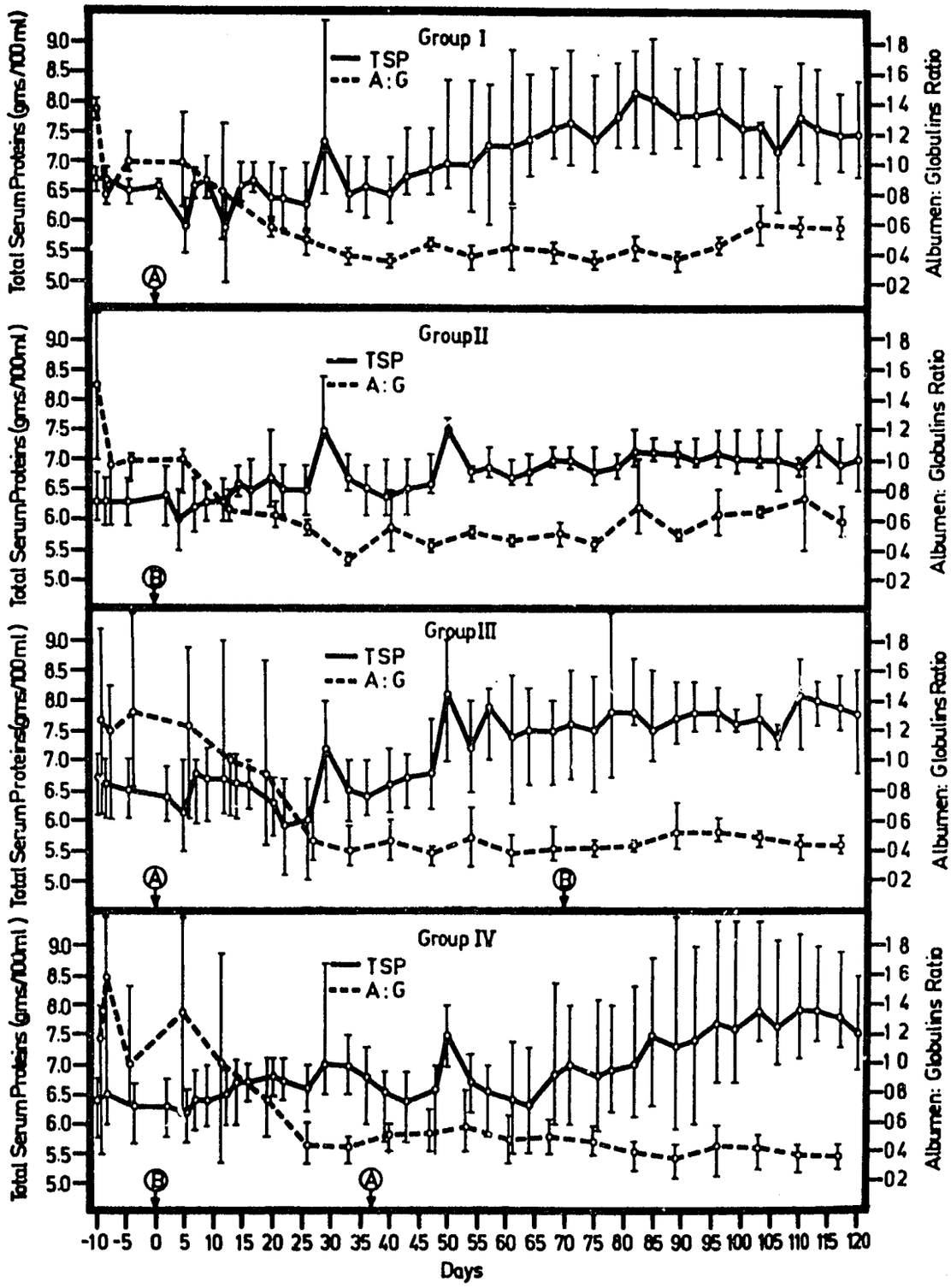
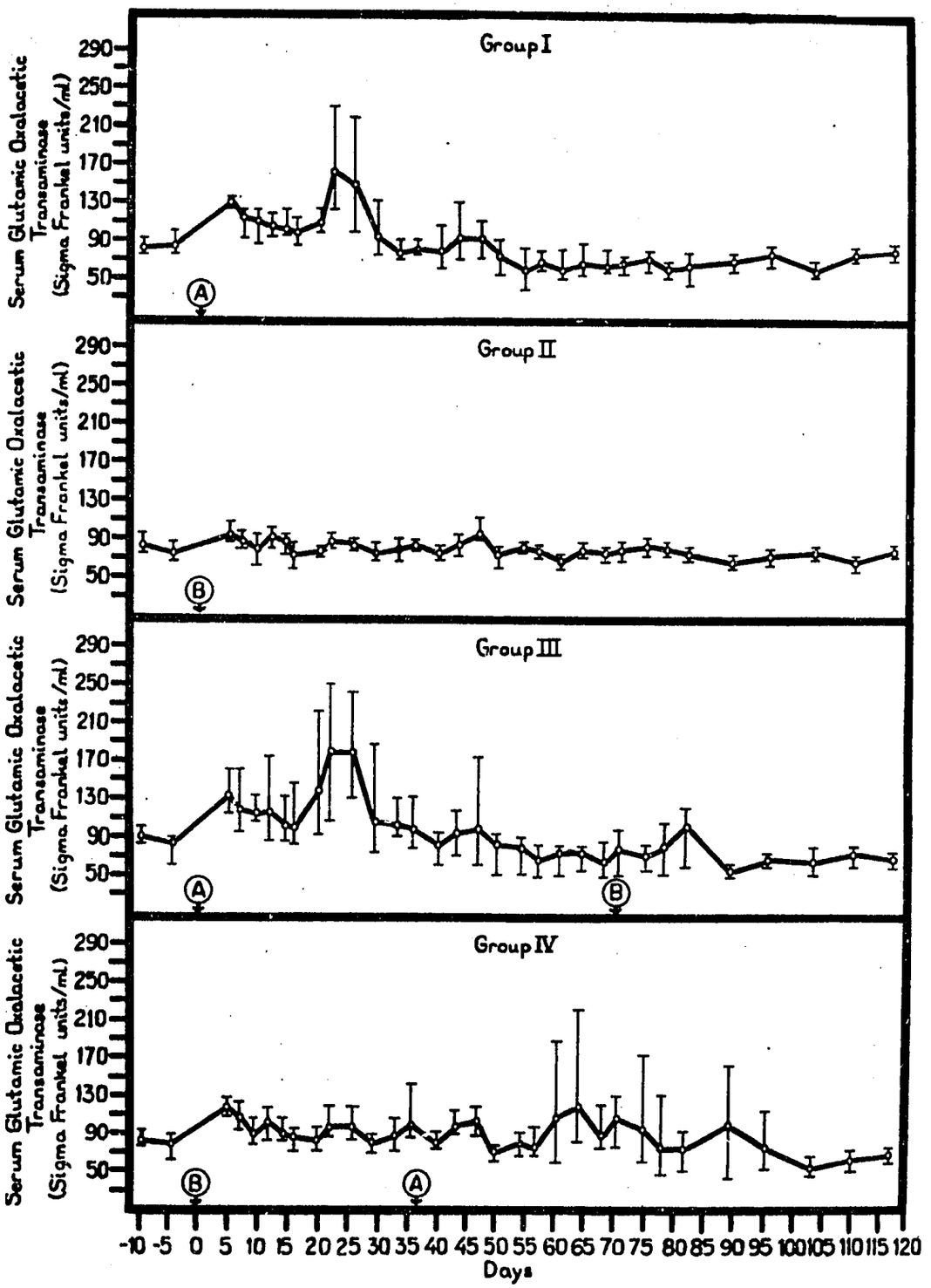

Group I
Group II
Group III
Group IV
TSP
A:G
Total Serum Proteins (gms/100ml)
Albumen: Globulins Ratio
Days
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120

Fig. 8. Serum glutamic oxalacetic transaminase levels of the 4 groups of calves experimentally infected with Anaplasma marginale and/or Babesia bigemina. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale (A) or with Babesia bigemina (B).


Group I
Group II
Group III
Group IV
Serum Glutamic Oxalacetic Transaminase (Sigma Frankel units/ml)
290
250
210
170
130
90
50
A
B
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
Days

range of 638 to 1972 units was observed as compared to an average preinoculation value of 486 units (Table 4). No increase was observed in serum sorbitol dehydrogenase in the calves of Group II. Serum sorbitol dehydrogenase level was elevated to 826 Sigma units/ml. with a range of 562 to 1346 units in the calves of Group III on day 110 PI, as compared to an average preinoculation value of 485 units. An elevation in serum sorbitol dehydrogenase was observed in the calves of Group IV on day 110 PI at which time a level of 1363 Sigma units/ml. with a range of 603 to 2018 units was observed, as compared to an average preinoculation value of 419 units.

Total, direct and indirect serum bilirubin. Slight increases were observed in total serum bilirubin in each of the experimental groups (Fig. 9). However, at no time did the total serum bilirubin exceed the normal reported range of 0.2 to 0.8 mg.%.[6] On day 33 PI a high value of 0.43 mg.% was observed in Group I, as compared to an average preinoculation value of 0.30 mg.%. The calves of Group II also showed a maximum elevation of total serum bilirubin on day 33 PI with an average level of 0.55 mg.% as compared to an average preinoculation level of 0.19 mg.%. A maximum elevation in serum bilirubin occurred in Group III on day 26 PI when it was observed to be 0.51 mg.% as compared to an average preinoculation level of 0.18 mg.%. On day 89 PI a maximum elevation of 0.64 mg.% was observed in the calves of Group IV. The average preinoculation level for the group was 0.25 mg.%.

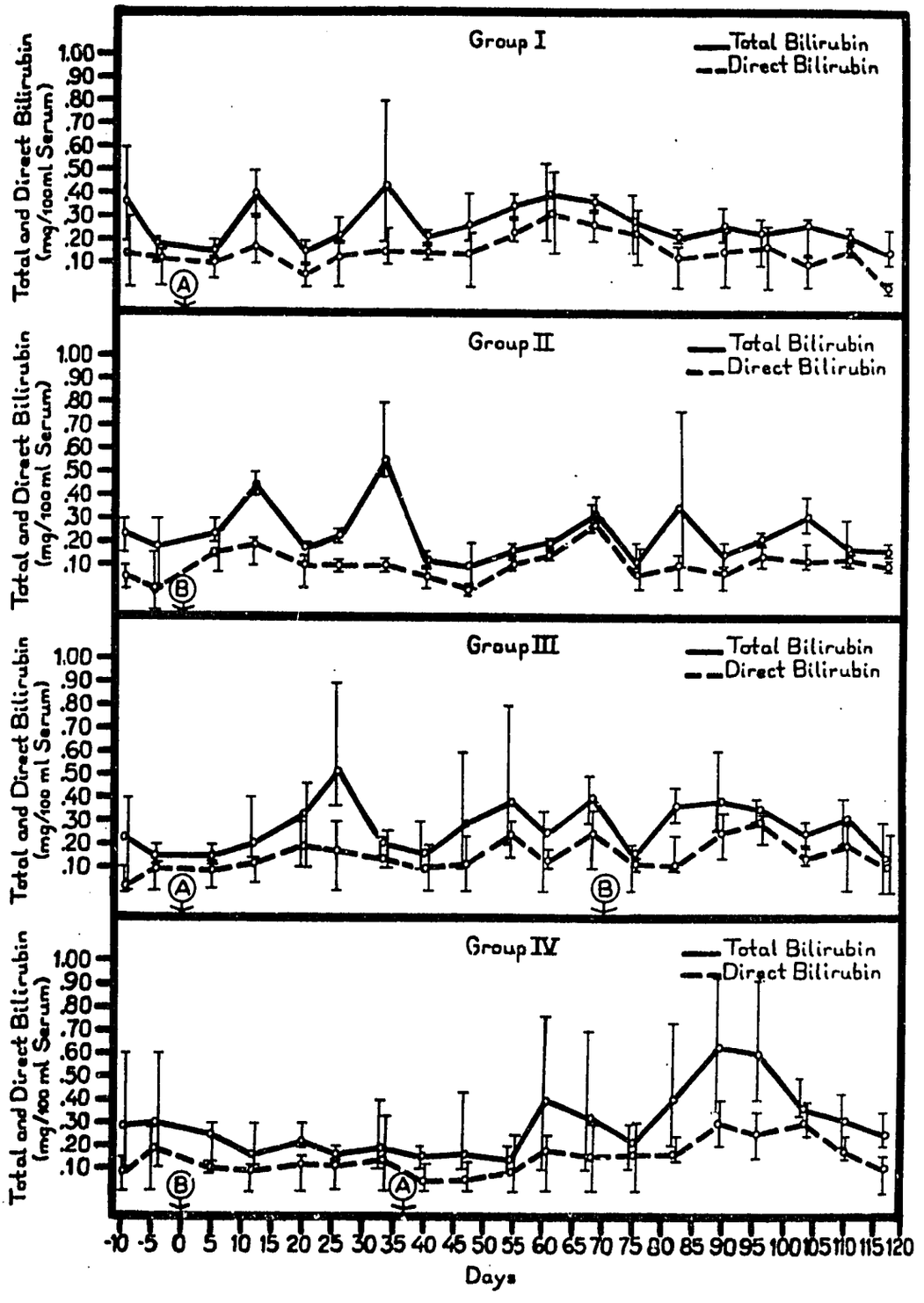
TABLE 4. Serum Sorbitol Dehydrogenase Concentrations of the 4 Groups of Calves Experimentally Infected with *Anaplasma marginale* and/or *Babesia bigemina*.

	DAY									
Groups*	-12	-10	+12	26	40	54	68	82	96	110
I	307	665	480	339	568	500	ND**	ND	634	1154
II	493	558	613	593	388	446	ND	ND	261	586
III	467	503	426	516	300	391	ND	ND	259	826
IV	423	415	411	666	296	299	ND	ND	333	1363

*Group means are presented in Sigma Units/ml. of serum

**ND -- Not determined

Fig. 9. Total and direct serum bilirubin concentrations of the 4 groups of calves experimentally infected with Anaplasma marginale and/or Babesia bigemina. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale (A) or with Babesia bigemina (B).


Group I
Total Bilirubin
Direct Bilirubin
Total and Direct Bilirubin (mg/100ml Serum)
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
A
Group II
Total Bilirubin
Direct Bilirubin
Total and Direct Bilirubin (mg/100 ml Serum)
B
Group III
Total Bilirubin
Direct Bilirubin
Total and Direct Bilirubin (mg/100 ml Serum)
A
B
Group IV
Total Bilirubin
Direct Bilirubin
Total and Direct Bilirubin (mg/100 ml Serum)
B
A
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
Days

Serum protein electrophoresis. A continual increase in the concentration of serum gamma globulin was observed in the calves of Group I starting on day 26 PI and continuing to day 82 PI when an average maximum concentration of 4.21 gms.% with a range of 3.65 to 4.76 gms.% was observed (Fig. 10). The concentration of gamma globulin decreased following day 82 PI until on day 120 PI a concentration of 3.14 gms.% was observed. The alpha and beta globulin fractions increased in Group I to maximum concentrations of 1.23 and 1.37 gms.% on days 20 and 26 PI respectively and then decreased to remain slightly above preinoculation levels thereafter (Fig. 10). The albumin:globulin ratio decreased below preinoculation values in Group I by day 12 PI and remained substantially below preinoculation values thereafter (Fig. 7). The serum albumin concentration was also observed to decrease and to remain slightly below preinoculation levels for the duration of the experiment (Fig. 11).

An increase in the concentration of serum gamma globulin was observed in the calves of Group II starting on day 5 PI (Fig. 10). The concentration of the gamma globulin fraction remained substantially above preinoculation values for the remainder of the experiment with an average maximum concentration of 2.84 gms.% and a range of 2.42 to 3.00 gms.% being observed on day 33 PI. The alpha and beta globulin fractions increased above preinoculation levels on day 20 PI and remained slightly above preinoculation concentrations thereafter (Fig. 10). The albumin:globulin ratio decreased below preinoculation values on day 12 PI and remained so thereafter

Fig. 10. The concentrations of alpha, beta and gamma serum globulins of the 4 groups of calves experimentally infected with Anaplasma marginale and/or Babesia bigemina. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale Ⓐ or with Babesia bigemina Ⓑ.

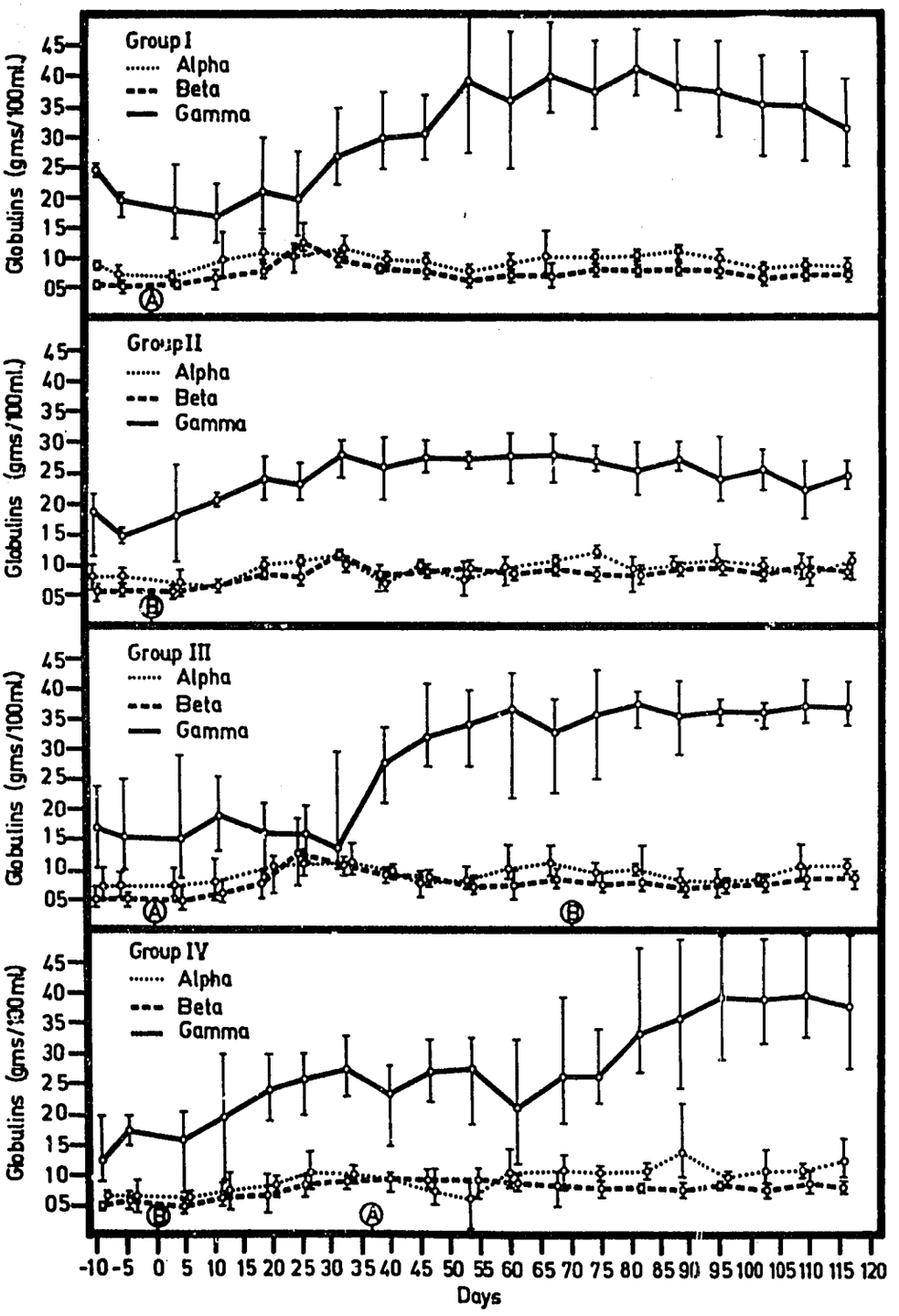
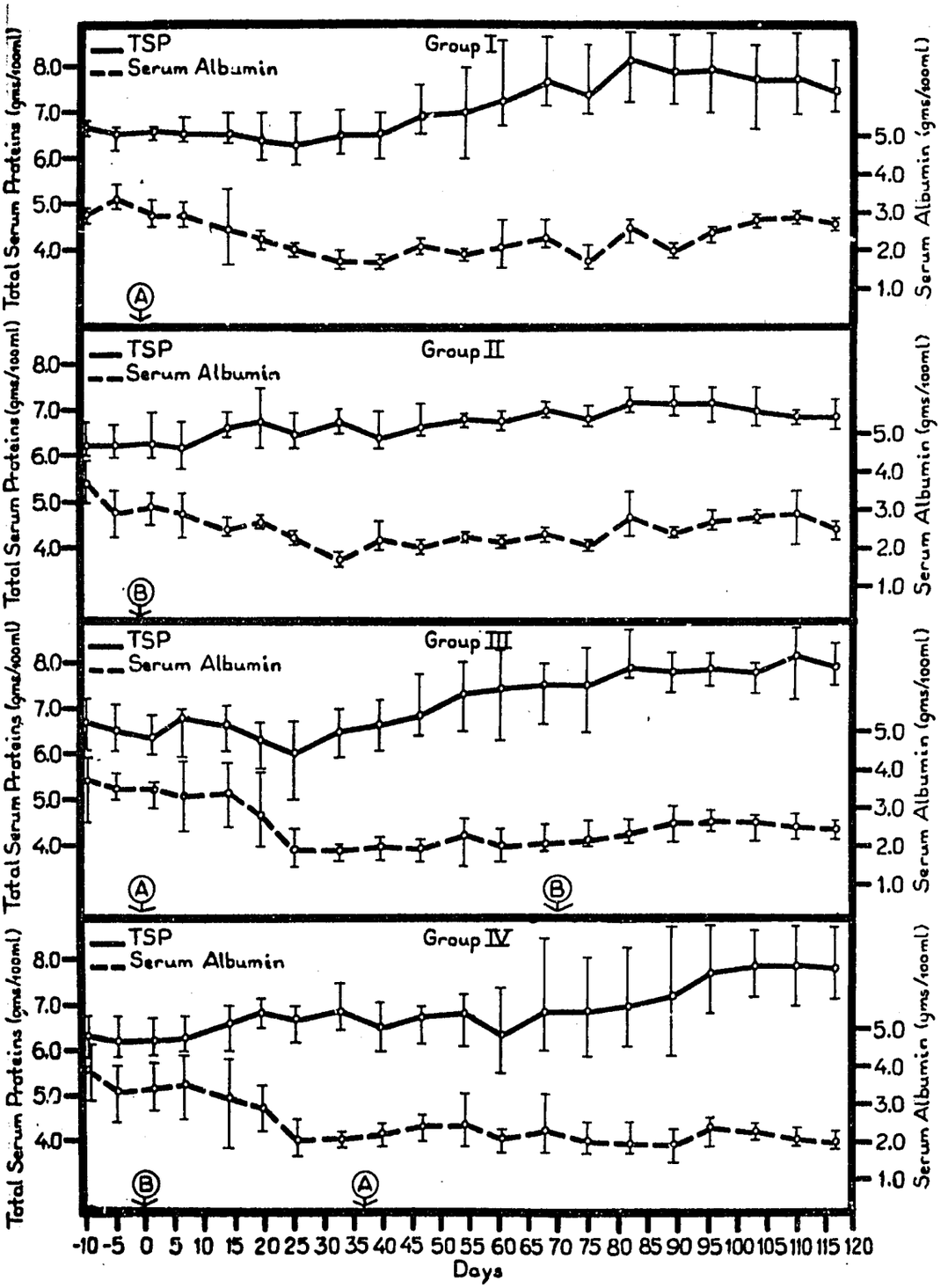

Group I
Alpha
Beta
Gamma
Globulins (gms/100ml)
Group II
Group III
Group IV
Days
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120

Fig. 11. Total serum proteins and serum albumin concentrations of the 4 groups of calves experimentally infected with Anaplasma marginale and/or Babesia bigemina. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale (A) or with Babesia bigemina (B).


TSP
Serum Albumin
Group I
Group II
Group III
Group IV
Total Serum Proteins (gms/100ml)
Serum Albumin (gms/100ml)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
A
B
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
Days

(Fig. 7). The serum albumin concentration decreased from an average preinoculation concentration of 3.27 gms.% to 1.70 gms.% on day 33 PI (Fig. 11). It then fluctuated between 2.03 and 2.87 gms.% for the remainder of the experiment.

An increase in the gamma globulin fraction was observed in the calves of Group III, following day 33 PI and continuing until day 82 PI, when an average maximum concentration of 3.70 gms.% and a range of 3.32 to 3.90 gms.% was observed (Fig. 10). The concentration of gamma globulin then decreased slightly and remained stable for the remainder of the experiment. The beta globulin fraction increased to a concentration of 1.34 gms.% on day 26 PI, after which it decreased to remain slightly above preinoculation levels thereafter. The alpha globulin fraction increased to maximum concentrations of 1.17 and 1.18 gms.% on days 33 and 68 PI respectively, remaining above preinoculation levels and above beta globulin concentration for the duration of the experiment. The albumin:globulin ratio decreased to below preinoculation values by day 12 PI and remained so thereafter (Fig. 7). The serum albumin concentration decreased in the calves of Group III from an average preinoculation value of 3.62 gms.% to a low concentration of 1.86 gms.% on day 26 PI (Fig. 11). The albumin concentration fluctuated thereafter between 2.00 and 2.64 gms.%.

An increase in the gamma globulin fraction was observed in the calves of Group IV starting on day 5 PI (Fig. 10). A momentary decrease in concentration was observed from day 54 until day 68 PI,

after which the concentration continued to increase to an average high concentration of 3.98 gms.% with a range of 2.86 to 4.90 gms.% on day 96 PI. The alpha and beta globulin fractions increased only slightly above preinoculation values with the alpha fraction increasing more than the beta fraction (Fig. 10). The albumin:globulin ratio decreased below preinoculation values on day 12 PI and continued to gradually decrease for the remainder of the experiment (Fig. 7). The serum albumin concentration of Group IV decreased from an average preinoculation concentration of 3.51 gms.% to a low concentration of 1.97 gms.% on day 89 PI (Fig. 11). Following day 20 PI serum albumin concentration fluctuated between 1.97 and 2.45 gms.%.

Bone marrow biopsies. Bone marrow specimens were collected on days 0, 50, 90 and 120; and myeloid:erythroid ratios were determined. The myeloid:erythroid ratio steadily decreased in each of the experimental groups (Table 5). The ratio was substantially lower in Groups III and IV on day 120 than in Groups I and II.

Serology.

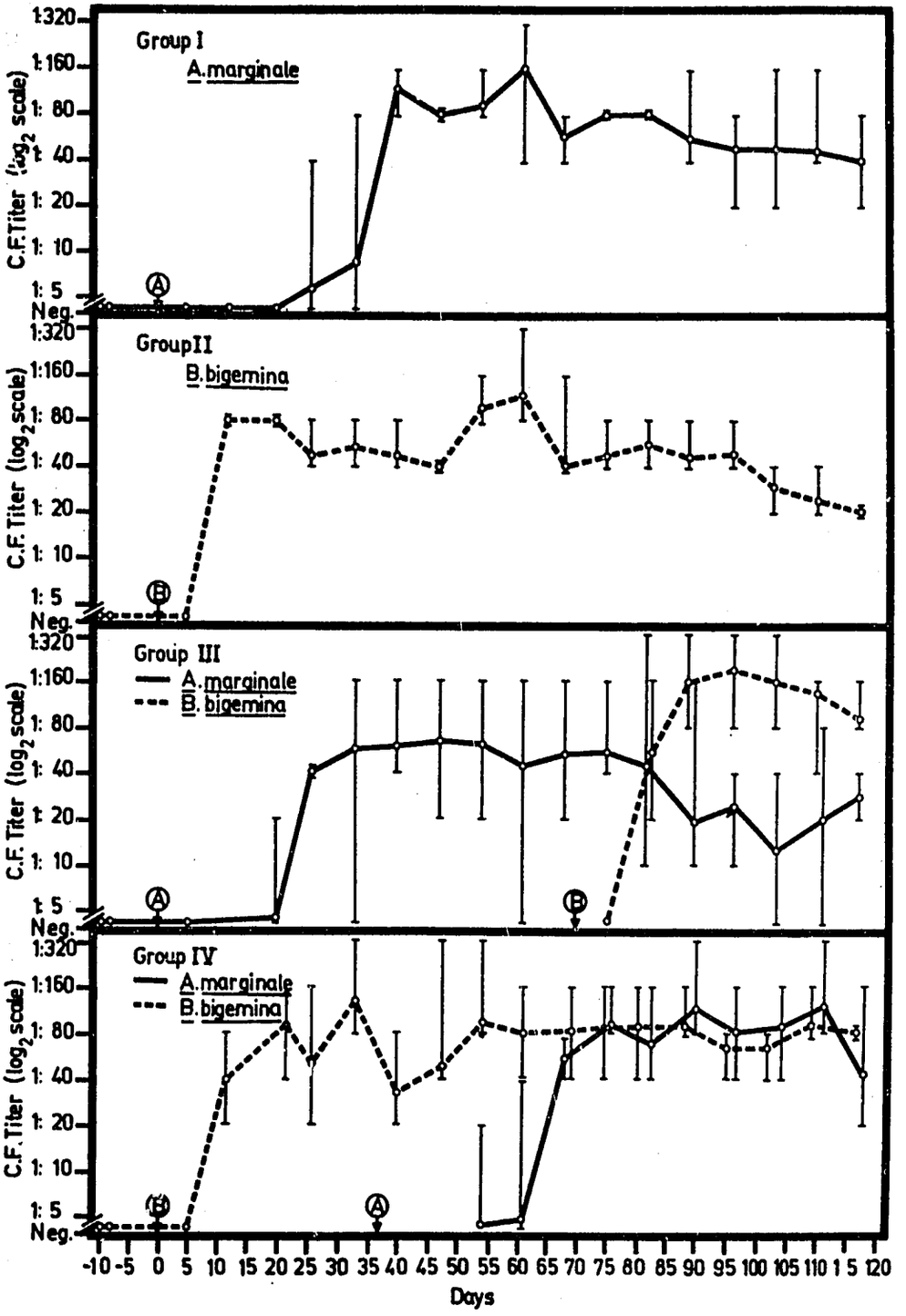
The complement fixation (CF) test for A. marginale and for B. bigemina was conducted on serum samples collected every seventh day of the experiment. Complement fixing antibodies for A. marginale were first observed in the calves of Group I on day 26 PI (Fig. 12). The average CF antibody titer increased to a maximum of 1:160 on day 61 PI and then decreased to 1:40 on day 120 PI. Complement fixing antibodies for B. bigemina were not observed in Group I.

TABLE 5. Myeloid:Erythroid Ratios of Calves Experimentally Infected with *Anaplasma marginale* and/or *Babesia bigemina* as Determined by the Examination of Bone Marrow Biopsies Collected on Days 0, 50, 90 and 120 of the Experiment.

Groups	Calf No.	M:E RATIO			
		Day 0	Day 50	Day 90	Day 120
I	122	0.59	0.23	0.40	0.57
	123	0.56	0.17	0.20	0.23
II	127	0.53	ND*	0.36	0.24
	128	0.60	ND	ND	0.64
III	133	0.78	0.25	0.15	0.18
	144	0.64	0.34	0.36	0.34
IV	147	0.49	0.30	0.14	0.13
	149	0.55	0.28	0.22	ND

*ND — Not determined

Fig. 12. Complement fixing antibody titers of the 4 groups of calves experimentally infected with Anaplasma marginale and/or Babesia bigemina. Group means are presented and vertical lines represent range. Encircled letters indicate time of subcutaneous inoculation with Anaplasma marginale (A) or with Babesia bigemina (B).


Group I
A. marginale
Group II
B. bigemina
Group III
A. marginale
B. bigemina
Group IV
A. marginale
B. bigemina
C.F. Titer (log2 scale)
1:320
1:160
1: 80
1: 40
1: 20
1: 10
1: 5
Neg.
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 1 5 120
Days